STUDIES ON CELL STRUCTURE BY THE FREEZING-DRYING METHOD

I. INTRODUCTION

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FOUR FIGURES

The important researches of Held (1895, 1897) on the structure of the nerve cell, those of A. Fischer (1899) on the fixation, staining and structure of protoplasm, of Bütschli (1892) on protoplasmic foams, and of Hardy (1899) on the structure of gels, marked a new epoch in the history of cytological research.

From a study of the surviving nerve cells from various sources and a comparison of the results so obtained with the appearances of nerve cells in fixed preparations treated in various ways, Held came to the conclusion that the familiar Nissl bodies of the nerve cell were not present as such in the living cell but on the contrary were products of a precipitation, by the reagents, of a substance which existed in the living cell in a state of solution diffused through the cytoplasm.

A. Fischer (1899), in an extensive study of the effect of commonly used histological fixing agents on protein solutions of various compositions, showed that by the action of these reagents sponge-like, filamentous and granular structures could be produced in otherwise homogeneous solutions.

Hardy (1899) showed that gels fixed under tension presented along the lines of stress filamentous structures resem-

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bling similar elements frequently described by cytologists in cytoplasm.

The skepticism engendered by these investigations concerning the structures demonstrated in protoplasm by histological methods put into disrepute not only the observations based on materials treated with the reagents in question, but also, undeservedly, the direct observations of Balbiani (1881), Flemming (1882), Carnoy (1884), and others on living cells. The whole granular theory of Altmann (1890) went for a time into the discard, since Fischer had produced by precipitation in solutions and stained successfully by Altmann's method just such granules and threads as the Altmann method demonstrated in cells. Altmann, unfortunately, had not availed himself of the opportunity to test his observations by reference to the living cell in which much of his objective findings could have been very easily confirmed.

At the very time these destructive criticisms were in preparation, Benda (1897), using a new method devised by himself, was resuming the study of the granules and filamentous structures described by Flemming and Altmann. He did not at first recognize that the structures which he was demonstrating were for the most part the same as Altmann's and he gave a new name to them, namely, mitochondria. He also repaired ('01) the defects in Altmann's observations by searching with success for these structures in the living cell.

This demonstration by Benda that the structures described by Altmann were not artefacts (as might be deduced from the experiments of Fischer) but realities was strengthened by Michaelis ('00) who, without the use of coagulating agents, saw similar structures in the living cell and discovered that they could be stained in the living cell by a non-coagulating dye (Janus green).

The descriptions of the studies of Benda and of Michaelis made, however, but little impression for a time. It became obvious, however, that although the filaments of Flemming and the granules and filaments of Altmann had emerged successfully through study of the living cell from attacks on

their reality as cell structures, much that had been won by the use of chemical methods of preservation was distinctly under suspicion. On the one hand, many investigators assumed a completely nihilistic attitude toward cell structure and preferred rather to deduce the structure of living matter from the behavior of colloids in solutions and gels than to look at it directly in an endeavor to find out what was real and what was false in the previous descriptions; on the other hand the universal skepticism concerning intracellular structures led to renewed efforts to penetrate the secrets of living substance by direct observation, and out of these efforts have grown the valuable results obtained by the study of living tissue by direct illumination and by dark-field study, the contributions derived from the cultivation of tissue in vitro, and the many valuable results of microdissection.

The new attitude, however, is distinctly one of analytic research; the old confidence in the reality of observations made by technical methods has largely disappeared. This is a real advance and we owe it wholly to the criticisms of the Fischer-Hardy school. The methods of tissue culture, and of microdissection in particular, have permitted not only the direct study of living cells, their growth, reproduction, and functional changes, but also have permitted the direct application to such cells of vital stains, of salt solutions of various composition and concentration to determine the osmotic properties and permeabilities of the cell and its components, and the external and internal application of reagents of various sorts to determine solubilities and reaction of cellular constituents. The methods of microdissection have also taught us much about the consistence and viscosities of intracellular elements.

It is obvious, however, that, with the aid of reagents and of vital stains, investigations made along these lines depend primarily upon visibility. They leave us completely in the dark as to whether optically empty and, as tested by microdissection, physically homogeneous cytoplasm possesses a chemical complexity beyond the range of ordinary visibility.

Much of value that has been revealed by the ordinary processes of histological research has failed to be substantiated by these direct methods. Matters of such importance as the distribution of chromatin in the interkinetic nucleus, the distribution of the important Nissl substance in the nerve cell, and that of many important substances recoverable by ordinary analytic methods have remained in doubt. A considerable degree of chemical heterogeneity may be possible without visible complexity of structure or obvious resistance to the microdissection tool.

The investigation of the distribution of chemical substances in cytoplasm has been beset with extraordinary difficulties. When reagents are applied to the living cell or the surviving cell the results are not easily interpreted, because the reagent may penetrate slowly and slowly achieve a considerable concentration in the cell. Moreover, the reagent is acting not on the individual constituents of the cell whose distribution is sought, but upon the cytoplasm as a whole, and the result is a composite one depending on the interaction of all these several factors. Moreover, the excitation of cytoplasm by even minute quantities of reagents employed in microchemical investigations may so disturb the equilibrium between the several phases of cytoplasmic structure that the distribution of substances in the cell as a whole is completely changed. For investigations which involve the application of agents which hydrolyze proteins, a disturbing factor not only in the location of the reaction but also in the distribution of the reaction product is introduced. The results of the application of Millon's reagent, for example, are modified by the presence of even traces of alcohol, and, of course, the presence of mercurial compounds must in all these cases remain under suspicion. Furthermore, the colored compound which is the result of this reaction passes through a series of changes proceeding from a molecular dispersed state to that of a particulate suspension. In the molecular dispersed phase it may shift from the point of origin to a suitable adsorbing surface and the character of the precipitations which have

been involved in the primary preparation of the material may readily change completely the picture obtained by the use of the reagent.

It is obviously necessary not only for the purpose of investigating the distribution of chemical substances in cells but also for the application of reagents themselves to develop new methods of investigation which permit the direct approach by chemical means to the study of cell composition with as little change in the state of constituent substances as is possible. Above all, these must be methods which avoid gross precipitation phenomena.

Such a method may be found in the Altmann freezing-drying method recently elaborated in this laboratory by Gersh ('32). This method was originally suggested by Altmann (1890) for the purpose of determining the reality of his granula. It was later tested by Bayliss ('24) and Mann ('02) who confirmed Altmann's statement that material frozen and dried by evaporation at a temperature of 20° below zero retained its form. They also perceived the possibilities of the method in the matter of microchemical investigations but failed to exploit them.

The difficulties of the original method of Altmann were such as to discourage all but the most devoted investigators. The method as recently elaborated by Gersh imposes no such difficult responsibilities on the investigator who exploits it. For this method in detail, the reader is referred to the original paper (Gersh, '32).

In brief, the method consists of freezing the tissue in liquid air immediately on removal from the body, transferring it rapidly to a chamber cooled to –20° C., in which, by the combined action of a Hy-vac pump and a mercury vapor pump, a vacuum is established which rapidly reaches 0.001 mm. of mercury and slowly rises, as the water is evaporated, to a vacuum of 0.0001 mm. of mercury, both measured by the MacCleod gauge. When the high vacuum has been maintained unchanged for a period of 12 hours, the preparation is considered completely dried. It is then embedded in melted

paraffin in vacuo; the time taken for this embedding is very short, 1 to 2 minutes. It is then cut into sections in the usual way, mounted on slides, avoiding water, and the section is now ready for further treatment.

Since the publication of this paper the method has been improved and extended. In the original experiments the cytological fixation was least satisfactory at the surface of the blocks, due to the formation of ice crystals. It has been found that by creating a new surface by placing the material to be investigated in a thin layer of another tissue, such as liver or muscle, much larger masses of satisfactory fixed histological material can be obtained, and the artefacts produced by ice formation in the tissue in general are diminished. It has also been found that in the case of mammalian tissues the condition of the material for investigation is much improved if the animal is kept without water for a day or two before the tissue is taken for study. This apparently reduces the amount of free water capable of ice formation in the tissues and so diminishes the ice artefacts.

The quality of the fixation obtained by this method is much better than might be anticipated and can be compared favorably in some cases with that obtained by ordinary histological fixatives. In figures 1 and 2 may be seen low power photomicrographs of two preparations of liver of Amblystoma, one fixed by the method indicated (freezing-drying method), and the other fixed by the formalin-Zenker method. The appearance of the former seems quite as acceptable a histological fixation as the latter. As a matter of fact, the sections of the dried material reproduce more closely the appearances as seen in the living cell. In the unstained preparations of this material the spaces occupied by the fat droplets, which are abundant in the fresh cell, are perfectly spherical and the cytoplasm, with its contained chromophile substance and mitochondria, and the glycogen, fill all the remaining space. In the formalin-Zenker material, on the other hand, in the process of fixation, the cytoplasm has withdrawn from the surfaces of the fat globules and made larger and less regular spaces

than are seen in the fresh cell. In addition, the glycogen has been driven over to one side of the cell, leaving the other side free from glycogen.

The sections obtained by the aid of this method, as indicated in the original paper, are directly available for testing the distribution of such substances as are microchemically detectable and for the application of reagents of various sorts to determine the nature and solubilities of intracellular constituents. Thin sections obtained by this method have experienced the action only of paraffin at a temperature corresponding to the melting point of the latter, and so may be presumed to have properties modified only to the extent which is possible by freezing and drying and the application of a temperature of 40°C. to 60°C., and the solvent action of the paraffin on cell constituents.

The effect of this process on the solubility of various protein substances can only be conjectured, yet by actual test the constituents of the blood plasma and of transudates remain completely soluble in salt solution. One cannot deduce from this solubility of plasma and transudate proteins the solubility of those of the cytoplasm, because that yet remains to be determined. Caution, however, must naturally be exercised in assuming that these solubilities are unmodified because it is a common experience of biochemists that proteins kept in a dry form lose their characteristic solubilities in water and salt solutions even when maintained at low temperatures. Such solubilities as are positive, however, by direct test under these conditions constitute a real advance in our knowledge of the cell, though the absence of solubility in water and salt solution cannot be interpreted as a primary insolubility in these media.

It is known as a result of the work of MacFadyen ('00) that organisms may survive freezing in liquid air, and Farmer ('00) showed that dried seeds were highly resistant to heat and still capable of germinating after the application of it. Farmer showed that completely dried egg albumin was not denatured by heating to a temperature of 110°C. for

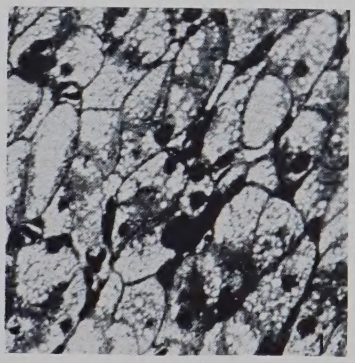
several hours, but when redissolved after this treatment still went into solution and had its coagulation temperature unchanged. On the basis of these observations one must be cautious in assuming without experiment that the submission to low temperatures is necessarily destructive of life, and one may expect to a degree varying with different tissues a retention of the properties of the materials as they exist in the living cell. To what extent this expectation may be realized, of course only actual trial will tell. It must be emphasized, however, that the operations must be performed with the utmost speed, particularly after the drying is accomplished, because of the gradual decrement in solubility of dried proteins.

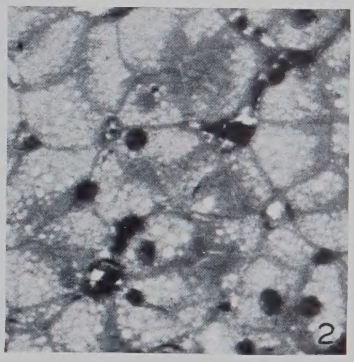
It may be assumed that the redistribution of substances in the cells frozen rapidly at a temperature of liquid air is only such as is determined by the formation of ice crystals. Apart from this pushing aside of the protoplasmic constituents by ice crystals, the distribution in the protoplasm itself will remain for the most part unaltered.

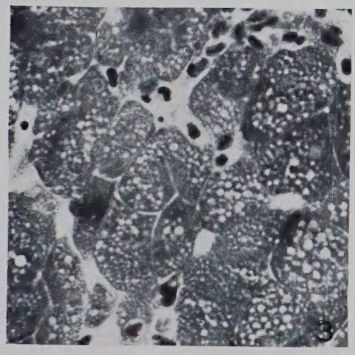
It is possible that such materials when reduced to thin sections after embedding in paraffin at once re-enter into solution on application of aqueous reagents, and that the result of the application of such reagents is really a combined result of re-solution and precipitation, the consequence being a redistribution of the material in the second phase of the process. In order to avoid errors on this basis, we have resorted to the device of fixing for purposes of control small pieces of the dried material by immersion in absolute alcohol or by the application of dry heat in vacuo.

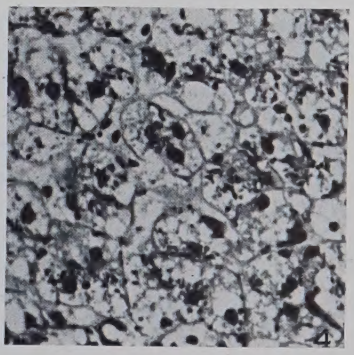
It is not to be expected that absolute alcohol acting upon completely dried material will produce the shiftings in cell constituents and the changes in cell structure that are associated with the fixation of fresh material in alcohol. Experience has borne out this expectation as is shown by the comparison of figure 3, which is a photograph of frozen-dried material denatured by immersion in alcohol, and figure 4, a photograph of material fixed directly in alcohol without dry-

ing. The disappearance of the characteristic cell constituents in the latter case is sufficient indication of the improvement induced by the new method. The application of alcohol to the dried material denatures the proteins in situ without changing their location, the tissue being altered only by the









Figs. 1 and 2 Low power photomicrographs of sections of the liver of Amblystoma stained with Hansen's hematoxylin and acid fuchsin. Figure 1, fixed by the freezing-drying method; figure 2, fixed with formol-Zenker, for comparison of the quality of fixation.

Figs. 3 and 4 Low power photomicrographs of the sections of the same liver stained with hematoxylin and Best's carmin. Figure 3, fixed by the freezing-drying method and later denatured in absolute alcohol. Figure 4, fixed directly in absolute alcohol to show the movement of glycogen associated with this method of fixation.

extraction and redistribution by adsorption of the constituents soluble in alcohol.

Furthermore, the dried material may be heated to a sufficient degree for a sufficiently long time to render the protein constituents of the cell insoluble. For this purpose the dried material is heated in a vacuum to between 130°C. and 150°C. continuously for a period of 2 to 24 hours. This may be presumed to alter the distribution of volatile substances and substances which have a melting point below the indicated temperatures. As a matter of actual test, the plasma of blood and the proteins of transudates may be quickly denatured by this method so that their solubilities in salt solution are completely abolished. As far as the protein constituents are concerned, the three methods check against one another, indicating that the distribution of substances in the undenatured sections is not due to re-solution and re-precipitation in a new place.

The sections of the undenatured material are difficult to handle because of the fact that water must be completely avoided in the fixing on slides, and for tests of chemical solubility it is necessary that they should adhere firmly to the surface of the slide. The sections of the denatured material, since they are to be used not for solubility tests but for morphological study and for microchemical tests, may be treated just as ordinary histological sections are.

These methods of investigation have been applied to a large number of different cytological problems, and the variations in the application of the method will be indicated under the separate topics as the results of investigation are described.

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STUDIES ON CELL STRUCTURE BY THE FREEZING-DRYING METHOD

II. THE NATURE OF THE MITOCHONDRIA IN THE HEPATIC CELL OF AMBLYSTOMA

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TWO PLATES (NINE FIGURES)

The conviction held by the majority of histologists that mitochondria are largely of a lipoid nature rests partly upon the hope that the elusive lipoids, which an analysis of all living substance reveals, have at last been morphologically identified and partly upon a considerable volume of circumstantial evidence. The latter has been summarized by Cowdry ('18) as follows:

(1) Mitochondria are almost completely soluble in alcohol, chloroform, ether, and dilute acetic acid. They are rendered insoluble by chromatization. They are not doubly refractile and they do not stain either in Sudan III or in Scharlach R. They are only sometimes blackened with osmic acid.

(2) It is said that part of the mitochondria substance is not soluble in these fat solvents and it is supposed that this portion is albumin.

(3) Artificial mitochondria have been made by Löwschin of lecithin in different salt and albumin solutions.

(4) The temperature solubility of mitochondria may also be significant. It has been discovered by Policard ('12) in the case of animal tissues and by N. H. Cowdry ('17) in plants that the mitochondria are soluble at a temperature from 48°C. to 50°C., while the other parts of the cells remain practically unaffected. Phosphatids have a low melting point also.

(5) There seems to be a certain correspondence between variations in the histological picture of mitochondria and the

variations in the phospholipin content of the same organ on chemical analysis. Thus Mayer, Rathery and Schaeffer ('14) have been able to alter the mitochondria experimentally in liver cells. In stages with more mitochondrial substance, a chemical analysis showed an increase in phosphorized lipoid; in stages with less, a diminution.

A consideration of these statements will show that there has not been in the work on this topic a clear distinction between solubility and melting point on the one hand, and loss of visibility on the other.

Evidence which has also had considerable weight in enabling histologists to reach these conclusions has been afforded by Regaud on the stainability of mitochondria after fixation in formalin. Regaud ('08) fixed materials in formaldehyde, submitted them to the action of potassium bichromate for a long period, and stained them in iron-hematoxylin. Under these circumstances the mitochondria stained well. If, however, the material after fixation in formaldehyde were extracted with fat solvents (such as alcohol, acetone, etc.) before chromatization, it was no longer possible to stain the mitochondria by the iron-hematoxylin method.

Similar observations have been made by Fauré-Fremiet, Mayer and Schaeffer ('10). The deduction from these observations was that a fatty component of the mitochondria had been extracted which was the substance specifically acted upon by the chrome salts.

These deductions as to the nature of mitochondria have been severely criticized by Arthur Meyer ('20), who says:

> The conclusion that the substance of the chondriosomes consists of an adsorption compound of fatty acids and protein body, it appears to me is not proven by the facts which Regaud and Fauré-Fremiet bring forward. The processes concerned in staining are of such a complicated nature that they do not permit such conclusions. Even the withdrawal of water by the fat solvent can produce in albumin gels and albumin sols a distinctive modification of the staining properties.

They have also been justly criticized by Wallin ('27), whose arguments in this connection have not received adequate recognition because of the general disbelief in his theory of the bacterial nature of mitochondria.

The attempt by Mayer, Rathery and Schaeffer ('14, '15) to establish a parallelism between the mitochondria content of the liver cell in section, as determined by microscopical examination, and the phospholipin content as determined by chemical extraction has made a great impression because it has the appearance of exact quantitative work. It should be pointed out, however, that the variation in the percentage amount of any chemical constituent of the liver cell would produce a coordinate but reverse change in the percentage of every other component. The most highly variable constituent, namely, glycogen, has not been determined in these analyses. No attempt has been made by the authors to establish a definite relation between unit of structure and unit of mass, which is absolutely necessary to make these analyses of any value whatsoever.

As regarding the solubilities indicated in a preceding paragraph, it must be pointed out that these solubilities, with the exception of certain observations of Fauré-Fremiet and of Chambers (to be referred to later), have been tested upon entire cells. When a reagent is applied to an intact cell it is not acting as such but is acting in combination with the constituents of the cytoplasm in which, if it is soluble, it achieves a slow increment of concentration. The molecular disturbances set up in the cell by this process have failed wholly of evaluation and we are confronted with the difficulty of distinguishing disintegration by molecular forces, a disappearance by solution, and a more or less rapid autolytic process going on in a protoplasm, stimulated, depressed, or otherwise altered by the action of the reagent. They are further complicated, as are the phenomena associated with the application of heat, by changes in refractive index which may cause structures to disappear from sight without actual destruction of them.

The disappearance of mitochondria at 48 to 50°C. as described by various authors has not been designated by any of them as a melting point, but a strong impression has been left that that is what they believed. Policard and Mangenot ('22) say:

> Between 48° and 50° the mitochondria undergo a sudden alteration. The precision of this disappearance can be utilized as a physical criterion for these bodies, a sort of temperature of melting for the cellular organs; no other of the elements morphologically visible in the cell undergo such a transformation.

The most that one can conclude from the results of all this experimentation is that mitochondria are highly labile constituents of the cell; all the rest is mere guessing.

As regards Löwschin's making artificial mitochondria out of lecithin and various salts and albumins, no conclusions can be drawn from these comparisons. Any one who examines under the microscope unripened photographic bromo-iodide emulsions will see an abundance of forms which resemble in size and shape the various kinds of mitochondria. Wallin ('27) also reports finding similar mitochondria-like forms in suspensions of proteins. But like Löwschin's structures, they are no more mitochondria than the soap foams of Bütschli are protoplasm.

Positive contributions of value concerning the nature of mitochondria are to be found especially in the work of W. H. and M. R. Lewis ('14, '15) and Chambers ('14, '24), in the earlier observations of Mott ('12) and Marinesco ('12) on the surviving nerve cell, and those of Fauré-Fremiet ('10) on Protozoa. These investigations are particularly notable because the authors have exercised extreme caution in avoiding unwarranted conclusions from the objective results of their observations.

The Lewises studied mitochondria in tissue cultures and noted particularly their variations in quantity, shape and position, and their responses to changes in osmotic pressure and to the direct or indirect application of chemical reagents.

Among their important observations are included the statement that mitochondia respond to changes in osmotic pressure even before their effect is apparent on the cell as a whole. In hypotonic solutions they increase in size and in hypertonic solutions they diminish in size; both reactions are reversible within certain limits. This observation shows that the mitochondria have a variable water content in equilibrium with the cytoplasm and that they are permeable to water both ways, while leaving in doubt the question as to whether the swelling in the hypotonic solutions (or the reverse) is the result of the presence of a semipermeable membrane on the surface of the mitochondria, of changes in lyophilic capacity of the organic substrate of the mitochondria, or of both. They found also that the mitochondria swelled in alkaline fluids and became beaded and converted into spherules in the vapors of acids. Both of these effects in their initial phases were reversible. The continued application of acetic acid vapor resulted in the complete disappearance of mitochondria from the cell.

Xylol, chloroform and ether, according to these authors, simply remove the mitochondrial material, or possibly dissolve the mitochondria and leave shadow forms or slight traces of degenerate mitochondria.

In their earlier work ('14, '15) they say that mitochondria are slightly blackened by osmic acid, although in a later statement ('24) the contrary is asserted. Much, of course, here depends upon what one means by 'blackened'; a slight change in the color in the direction of blackening might be interpreted as a reaction without, however, having any great significance. Similar references to the action of osmic acid have been made by numerous writers.

Chambers studied the mitochondria particularly thoroughly in the germinal cells of the grasshopper ('24). Microdissections of this material were accomplished in natural lymph collected from the basal joint of an amputated leg. The material mounted in this fluid was observed in the moist chamber. He says:

When cells burst the mitochondria show themselves more glistening than before. And when the cytoplasm dissolves the mitochondria persist in the surrounding fluid for a long time after the complete disintegration of the cytoplasm and of the nucleus. When the spindle of the metaphase is formed, the mitochondria assemble around it in a loose network. If the cell is destroyed at this moment by a needle, the network persists with the chromosomes dispersed in the interior which becomes fluid.

Spermatozoa which came into contact with this network only succeeded in freeing themselves by giving violent vibrations of the tail, and in some instances the material was so sticky that the sperm was retained until the viscosity of the material had diminished during the process of dissolution. In a case where one of two daughter cells was destroyed, the mitochondria filaments persisted as a bundle of filaments projecting outside the other daughter cell. Little by little they retract on all their length. This is associated with a shortening of the bundle, the extremities of the filaments tending to separate from one another and to roll up in the forms of sorts of curls. After the second cell was destroyed, the bundle of mitochondria persisted long enough to fuse at last into an amorphous granular mass.

The final fate of the mitochondrial substance in the tissue lymph is not indicated, but the facts brought out indicate a slow progress toward solution.

Marinesco ('12) studied the colloid granules visible in the dark-field in the fresh spinal ganglion cells and other nerve cells of mammals with the aid of the paraboloid condenser and tested their reaction to the application of different reagents. These colloid granules we now know are for the most part mitochondria, although those seen by Marinesco also included minute lipoid droplets. He found that in distilled water the cells increased in size and that the colloid granules were first separated owing to the solution of the cytoplasm and finally vanished. A solution of the colloid granulations, particularly in the periphery of the cell, was also procured by the use of alkaline solutions and of sodium and potassium iodides.

Mott, in the same year ('12), repeated these observations. He suggested that the granules might correspond with Altmann's bioblasts and agreed with Marinesco that they might also be identical with the neurosomes of Held. His results are somewhat different from those of Marinesco as regards the action of water and weak alkalis. He says:

If the living nerve cells are subjected to the action of water or a weak 1 per cent solution of ammonia an endosmosis takes place, the cells swell up, water passes through the cell membrane and collects in globules; it does not seem to dissolve the granules, but to be absorbed by the homogeneous spongioplasm; and as the process continues, semi-opaque fluid droplets appear within the cell and the granules escape into the surrounding medium, where they exhibit active Brownian movements on the dark ground. The granules are extremely minute (less than 1 μ); they sometimes appear spherical, sometimes oval, in shape. It appears to me that they consist of a colloidal fluid surrounded by a membrane possibly consisting of lipoids.

The observations of Chambers constitute at the present time almost the only data that we have on the behavior of mitochondria when extracted from their native cytoplasm and observed without drying, and even in these observations it is impossible to avoid the suspicion that spontaneous changes of the nature of an irreversible gelation are contending in rate with the tendency to go into solution in the tissue lymph.

The earlier studies of Fauré-Fremiet ('10) on the behavior of mitochondria when liberated from the cell in the Infusoria, especially in Paramoecium, show that under these circumstances their response is quite different from that in intact cells. He found among other things that mitochondria of Paramoecium brought into contact with water immediately swell. This result was also obtained by treatment with hypotonic solution or by an alkaline medium. Alcohol, ether and chloroform in a very weak solution did not produce the same results. He found that mitochondria isolated upon a slide by tearing an infusorian apart took a slight grayish color with osmic acid, which, however, became much more intense

if the material were afterward treated with pyrogallic acid. If one treated the mitochondria so spread on the slide first with alcohol, chloroform, ether or acetone at the temperature of the laboratory, or even in an oven at 40°C., the staining with osmic acid could not be obtained, whence he concluded that the solvents employed had dissolved an osmiophile substance fixed upon the mitochondria. But, he adds, "They do not dissolve the mitochondrial granulations which always persist and can then be easily colored by a large number of stains, particularly fuchsin, eosin, and so forth."

For our observations on mitochondria, the liver cell of Amblystoma has been chosen because of the splendid size of the mitochondrial elements, and because of the fact that this particular tissue element is extraordinarily well fixed by the freezing-drying technique. The observations have been made on sections embedded (as indicated above) in paraffin, cut into sections of 4 μ, and mounted on slides thinly coated with Mayer's albumin fixative. The sections are pressed down onto the surface of these slides, then warmed to the melting point of paraffin. The paraffin is removed by means of abundant petroleum ether, which is finally allowed to evaporate from the section, and the reagents are applied directly to the dried section. After the application of the reagents, the section is stained with Hansen's chromalum-hematoxylin, followed by anilin acid fuchsin, which is differentiated by Altmann's picric acid-alcohol method. In this method without any previous chromation, excepting such as is afforded by the small chromalum component of the hematoxylin, the mitochondria are stained brilliantly with the acid fuchsin.

Inasmuch as a certain measure of change may be conceived to take place in the dried sections of the undenatured material immediately on the application of aqueous reagents, the sections obtained by this method have been compared with sections of similar material denatured by heat in vacuo or by absolute alcohol after drying.

To test the solubility of the dried mitochondrial remains in various fat solvents, the material has been placed in a

Soxhlet apparatus and extracted by means of the fat solvents for 44 to 60 hours, then embedded in paraffin, sectioned and stained. This process of extraction by the fat solvents also denatures the proteins of the tissue so they may be treated very much as an ordinary fixed preparation.

The sections obtained by these methods afford the first opportunity for the application of chemical reagents directly to the dried organic basis of mitochondria.

In figure 10 is shown a section of two cells of the liver of Amblystoma prepared according to the method described above. It has been stained with Hansen's chromalum-hematoxylin and with anilin acid fuchsin; the latter differentiated by picric alcohol. In this figure the places occupied by the fat droplets are indicated by cavities. The mitochondria are brilliant red and the chromophile substance blue. The nuclear detail is not apparent because both the chromatin and the karyolymph stain with the hematoxylin.

The application of water or of salt solutions to undenatured, dried, thin sections of Amblystoma liver is not always followed by the same result. In some instances the application of water or salt solution results in the rapid removal, in the course of a few minutes, of all the mitochondria from the cell. The intermediate phases of this removal are shown in figure 11, in which is illustrated a cell of the Amblystoma liver treated for a brief period with distilled water, then stained in chromalum-hematoxylin and acid fuchsin. In this figure the chromophile substance is unaffected but the mitochondria are reduced to palely staining rings, much as one sees them in the deeper layers of a tissue fixed in a solution that contains acetic acid. The next phase (which is not illustrated) is complete withdrawal of the mitochondrial material. The stages of this change are as follows: The mitochondria first become beaded; in the later ones they break up into spheres which in time become vesicular, and finally dissolve. In these cases there is also an evident leeching out of substances from the cytoplasm. In other instances the mitochondria remain wholly refractory to water and salt solution and

may be treated by them for a considerable time without change of shape or loss of substance.

The reasons for these differences of behavior are not apparent, but it seems probable that the mitochondria possess a primary solubility in water and salt solution but are susceptible to a rapid and irreversible change into an insoluble form. The temperature of embedding does not seem to be a factor in this change since materials embedded in paraffin at a melting point of 40°C. and cut over a special cooling chamber behave exactly like those embedded at 60°C. It is evidently not a heat coagulation point. On the other hand, the length of time that the material is kept in the dry form in a desiccator seems to be of importance, since the mitochondria are more easily dissolved in water after short periods than after longer periods of preservation in the dry form.

The solubility of mitochondria in acetic acid, which has been maintained by practically every investigator who has worked on this topic, is not confirmed for the dried mitochondrial substance. Sections of undenatured material treated with acetic acid of 0.15 to 5 per cent for a period of 5 minutes at room temperature, or at 37°C. or at 55° show no change. Figure 13 shows a liver cell of Amblystoma treated with 1.2 per cent acetic acid for 10 minutes at room temperature. In this instance neither the mitochondria nor the chromophile substance is affected.

Treatment of the undenatured sections for 5 minutes with solutions of sodium carbonate, ranging in strength from 0.5 to 2.5 per cent also produces no change in the mitochondria.

Blocks of frozen-dried material were treated at a temperature of 140° in vacuo for a period of 4 to 22 hours, then embedded in paraffin and sectioned and stained as usual. According to the observations of Policard and Mangenot and N. H. Cowdry, the mitochondria disappear from the surviving cell when the temperature is raised to between 48 and 50°C., and it has been suggested that this resembles a melting point for the mitochondria. Heating to 140°C. neither changes the form of the mitochondria nor disperses the substance of

them. Figure 5 shows for comparison the mitochondria in a photomicrograph of an untreated preparation. Figure 6 shows a similar preparation of material which has been heated for several hours in vacuo to 140°C.

To test the effect of the so-called fat solvents on mitochondrial substance, pieces of the frozen-dried material were continuously extracted for 50 hours in a Soxhlet apparatus with the following reagents: alcohol, chloroform, acetone, sulphuric ether, petroleum ether. The material so extracted was then embedded in paraffin and sections cut and stained for mitochondria. Figure 7 shows a portion of a liver cell so extracted with acetone. Figures 8 and 9 show the result of similar extraction with sulphuric ether and petroleum ether. The extraction with alcohol and chloroform yielded similar results. These figures may be compared with figure 5, which represents an unextracted, undenatured preparation (see also fig. 10). It will be seen by reference to these photomicrographs that no one of the solvents mentioned produces any change in the form of the mitochondria or in the apparent amount of organic residue representing them. The inspection of these figures will show that the treatment of the dried material with these solvents has produced no recognizable removal of material from the mitochondria.

It is, of course, impossible from these experiments to say that no lipoid substance has been extracted from these structures, and it is also possible that the extraction in the hot solvents has failed to break up a firm protein-lipoid combination, but in any case the extraction produces no more visible change in the mitochondria than the extraction of the lipoid constituent produces in the cytoplasm. Lipoid capable of extraction by these solvents is not present in the mitochondria in sufficient quantity to modify perceptibly their appearance in the extracted materials. Moreover, in this process of extration in the Soxhlet apparatus, by means of alcohol, and so forth, no apparent change has been made in the stainability of the mitochondria. The assumption that the stainability of the mitochondria is due to the lipoid content is therefore untenable.

One of the difficulties in interpreting the mitochondria as of protein nature has been the almost uniformly negative result of the application of specific group color reactions for the proteins. Of these, of course, the most suitable is Millon's reagent, and the result of this has been almost uniformly negative. Noel ('22) and Giroud ('25), however, report obtaining a stronger Millon's reaction in portions of the cell where mitochondria are more abundant than in other parts of the cell, indicating the possibility that the mitochondria might have participated in the production of this intensity.

A possible reason for the negative result has been the unsatisfactory quality of the Millon's reagents employed in the tests. In order to have a Millon's reagent which is satisfactory for microchemical uses it is necessary to have one which acts progressively without the application of heat and in which the reaction does not readily pass over into a yellow phase on prolonged application. Such a reagent should give an almost immediate reaction with tyrosin in vitro, rapidly deepening to an intense red color and precipitating in the course of a couple of hours a red precipitate which should not change to yellow in a subsequent 24 hours. Millon's reagent made by the original and prevalent process of dissolving mercury in nitric acid does not often produce such a reagent.

A Millon's reagent having the required properties may be made as follows: A solution of nitric acid (40 per cent by volume) is obtained by making up with water 400 cc. of concentrated nitric acid, specific gravity 1.42, to 1 liter. This is allowed to stand for 48 hours and then is further diluted by adding one part to nine of distilled water, giving an approximate 4 per cent solution of nitric acid. The 4 per cent solution of nitric acid is then saturated with crystals of mercuric nitrate, which are added in considerable excess and allowed to remain, with frequent shaking, in contact with the nitric acid for several days in order to insure complete saturation. The solution is then filtered. To 400 cc. add 3 cc. of the original 40 per cent solution of nitric acid and 1.4 gm. of

sodium nitrite. This Millon's reagent has been used by one of us (R.R.B.) for many years and numerous tissues have been tested as regards the reaction of mitochondria with uniformly positive results. In the mitochondria of the hepatic cell of the frog, the Millon's reaction was particularly strong, but in cells where the mitochondria are small a reaction was obtained such as that described by Noel and Giroud, namely, the Millon's reaction, without being specifically referable to the mitochondria, was stronger in the areas of the cytoplasm where the mitochondria were more abundant. The results of these studies have not been published because of doubts as to the effect of fixing reagents upon the results, but the application of the reagent to the section prepared by the freezing-drying technique fully confirms the former observations.

In applying this solution to sections, the section fastened to the slide without the use of water may be immersed in the solution in the cold. A maximum reaction should be obtained in about 3 hours, after which time the section should show on the whole a perceptible rose color. It is better, of course, to put a number of slides in such a solution in a Coplin jar and to remove one from time to time for examination in order to obtain the maximum of reaction. When they show a perceptible rose tint, the examination may begin.

For this purpose the slides are removed from the Millon's reagent, immediately dipped in a 1 per cent solution of nitric acid, then passed rapidly through absolute alcohol and xylol and mounted in balsam. The purpose of the nitric acid washing is to avoid the precipitation of the basic mercuric nitrate on the slide, obscuring the results of the experiment. Sections may also be examined in glycerine.

Sections of Amblystoma liver treated with this reagent give a perceptible postive Millon's reaction in the mitochondria. As a matter of fact, the intensity of color in the mitochondria is greater than in other portions of the cell, either because the concentration of protein in these elements is higher or because the contained protein has a higher percentage content of the oxyphenyl radical which is necessary for the reaction.

The application of the xanthoproteic reaction has not so far been successful because of the destructive action of the nitric acid on the tissue.

In view of the demonstration contained in the preceding paragraphs that the mitochondria of the hepatic cell of Amblystoma are not chiefly lipoid in nature, that they have a primary solubility in water and salt solutions and that they have an actual protein content as indicated by Millon's reaction, it might be anticipated that they would also be removed by digestion with artificial gastric juice. This is in fact the case. If sections of undenatured material, fixed to slides, are placed for about 30 minutes in artificial gastric juice in 0.3 per cent hydrochloric acid at 37°, the mitochondria are completely removed. That this is a digestion and not a mechanical removal may be seen by reference to figure 12, which represents a cell so treated. In this figure the protein droplets of Berg, or, as we prefer to call them, the chromophile bodies, are not removed by this treatment, but the mitochondria have wholly disappeared and there is much loss of substance from the cytoplasm. The mitochondria are also rapidly removed by artificial pancreatic juice, but this is associated with more extensive destructive action on the cytoplasm as a whole.

The observations recorded in the preceding paragraphs show that the mitochondrial substance is not soluble in acetic acid or in alkalis, or in alcohol, chloroform, acetone, sulphuric ether or petroleum ether. They show, furthermore, that the mitochondrial substance does not melt at a temperature of 4 8to 50°, as has been supposed from the results of the application of such temperatures to surviving cells. The main mass of the mitochondrial substance is protein in nature, as indicated by the postive outcome of the Millon's reaction and by the digestibility in artificial gastric juice. The observations are contrary to all the preceding observations on the chemical nature of mitochondria except those of Fauré-Fremiet on the mitochondria of Infusoria liberated on a slide by breaking the cell. The reason for these contradictory re-

sults is obviously to be found in the fact that now for the first time the solubilities of mitochondrial substance have been tested actually on the substance itself. The results previously obtained by the application of reagents to intact cells have been in no sense solubilities but have been the results of a complicated series of reactions in which not only the solvent and the mitochondria but also the surrounding cytoplasm participated. The application to fresh tissue of those reagents which have been said to destroy mitochondria is in a sense an ineffective method of fixation of the cell, and the results include not only the direct effects of the reagent, but the slow autolytic processes which take place in the cell when left to itself.

The slight staining with osmic acid, which is described by practically all observers, is in itself no indication of a lipoid content. Osmic acid is far more versatile in its capacity for oxidation of organic materials than the biological literature would lead one to suppose. This gray reaction of osmic acid may mean no more than a greater concentration of protein substances in the mitochondria than the surrounding protoplasm. On the other hand, the staining of mitochondria by osmic acid may not be even a reduction, as it has been shown by Owens and Bensley ('29) that osmic acid reduced elsewhere in the cell may be deposited by adsorption on mitochondria and other bodies.

Fauré-Fremiet, Regaud, Mayer, Rathery and Schaeffer, and others, have laid great stress upon the effect on the staining reactions of mitochondria of previous extraction with alcohol, acetone, chloroform, and so forth. The failure to stain with iron-haematoxylin after extraction of these bodies, unless they were previously treated with chrome salts, has been interpreted as an indication that some soluble constituent had been removed from the mitochondria. Arthur Meyer has correctly pointed out that this deduction is by no means justifiable since the changes in stainability may just as well be due to changes in the consistence of the mitochondrial body as due to changes of a purely chemical nature. As pre-

viously stated, the action of fat solvents on dried material is not associated with any observable change in the staining properties of mitochondria.

Although our results do not justify the conclusion that the mitochondria contain no lipoid constituent, nevertheless it is apparent from results obtained that the localization of lipoid in the cell is at present impossible, and anything that might be said in this connection is equally applicable to the cytoplasm in general.

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PLATE 1

EXPLANATION OF FIGURES

5 to 9 High power photomicrographs of portions of liver cells in sections of liver of Amblystoma, fixed by the freezing-drying method, treated as described for each figure and stained with Hansen's hematoxylin and aniline acid fuchsin.

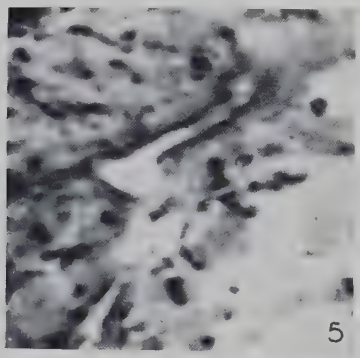
5 Unextracted, undenatured, stained directly.

6 Heated at 140°C. in vacuo for several hours.

7 Extracted in a Soxhlet apparatus for 50 hours.

8 Extracted with sulphuric ether in a Soxhlet apparatus for 50 hours.

9 Extracted with petroleum ether in a Soxhlet apparatus for 50 hours.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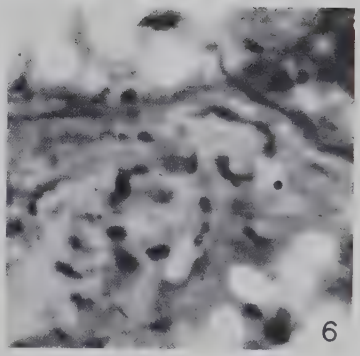
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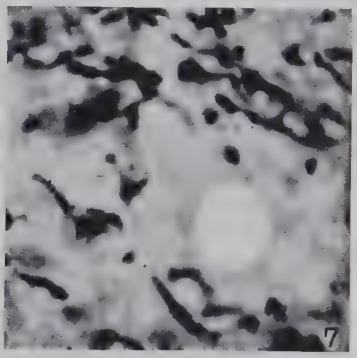
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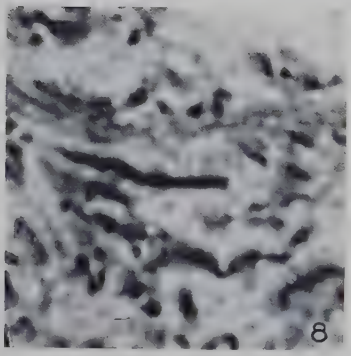
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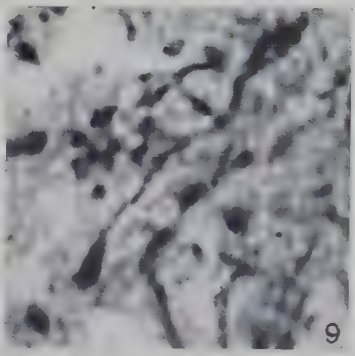
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PLATE 2

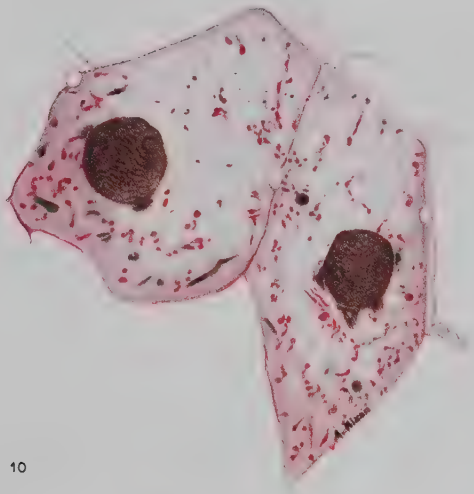
EXPLANATION OF FIGURES

10 Two liver cells of Amblystoma fixed by the freezing-drying method and stained with Hansen's chromalum-hematoxylin and anilin-acid-fuchsin, without the application of any previous reagent. Mitochondria are red; chromophile substance blue. × 960.

11 Same preparation as figure 1, but section was treated with distilled water for 5 minutes before staining. Mitochondria are in the form of rings and ovals; substance largely removed. Chromophile substance unaffected. × 960.

12 Same mode of preparation as in figure 1, with the exception that this figure has been treated with artificial gastric juice at 37°C. for 30 minutes before staining. Mitochondria are completely removed; chromophile substance unaffected, and much removal of substance from the cytoplasm. × 960.

13 Same preparation as figure 1, with the exception that the section was treated with 1.2 per cent acetic acid for 10 minutes at room temperature before staining. No effect on mitochondria or chromophile substance. × 960.



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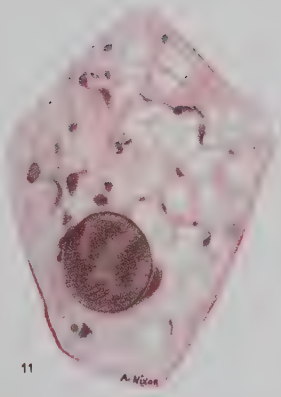

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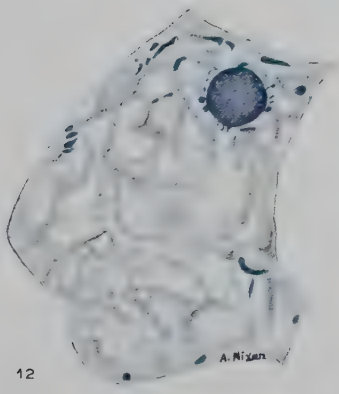

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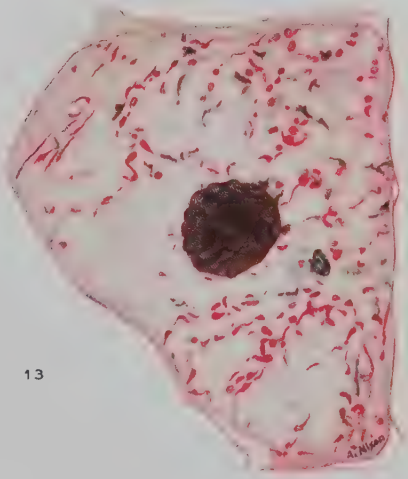

13

SOME OBSERVATIONS ON LIVING EGGS AND BLASTULAE OF THE ALBINO RAT

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NINE FIGURES

INTRODUCTION

For the successful experimental study of the embryology of mammals it is important to be able to cultivate the eggs outside of the maternal body. Brachet ('12, '13) cultivated rabbit blastocysts in vitro using pure, spontaneously clotted homologous plasma. He succeeded in obtaining the development from the primary tridermic stage (corresponding to vesicles of $5\frac{1}{2}$ days) to the secondary tridermic stage with formation of the primitive streak and chorionic villi. The ectoplacenta differentiated in vitro earlier than in situ. Its differentiation is not necessarily dependent upon stimuli either from the uterine mucosa or from the deciduomata.

W. Lewis and Gregory ('29) have succeeded in obtaining the cleavage of fertilized rabbit eggs in vitro, from the pronuclear to the later blastocyst stage, with formation of trophoblast, segmentation cavity and inner cell mass. These results show that rabbit eggs can segment outside of the maternal body. More recently Gilchrist and Pincus ('32) have studied the reactions of the rabbit egg outside the body.

In the rat, as in the mouse, Long ('12) has succeeded in obtaining artificial fertilization in vitro, but cytolysis occurred shortly after the formation of the second polar body.

[1] Visiting Rockefeller Fellow from the Istituto Anatomico della R. Universita di Milano (Italy). On account of Doctor Defrise's return to Milan, the manuscript has been revised by Dr. J. S. Nicholas.

No attempts to culture the blastula and gastrula have been made so far, although the rat is quite suitable for operations on embryos and foetuses (Nicholas, '25). By making daily systematic microscopic examinations of the vaginal smear in order to determine the progression of the oestrous cycle, it is possible to mate the females at the right time. When the females with regular cycles are selected, mating is almost always followed by fertilization when the microscopic examination of the vaginal smear shows clumped and well-preserved epithelial cells (second period).

I wish to express my thanks to Prof. J. S. Nicholas for the suggestion of the problem and to Prof. R. G. Harrison for the facilities of the laboratory.

OBSERVATIONS ON LIVING EGGS AND BLASTULAE

Previous data upon the dimensions of rat eggs may be summarized as follows: Sobotta and Burckhard ('11) (fixed material), ovarian ova: 60 to 65 μ, tubal ova: 55 to 60 μ; Kirkham and Burr ('13) (living material), unfertilized ova: 79 μ; Huber ('15) (fixed material), pronuclear stage: 70 by 62 μ. My observations on living material in the best medium available give the following: unfertilized, 86 by 79 μ; pronuclear stage, 80 by 75 μ; bicellular stage, first cell, 58 by 48 μ; second cell, 54 by 45 μ; tricellular stage, first cell, 52 by 43 μ, second cell, 45 by 40 μ; third cell, 40 by 35 μ; tetracellular stage, first cell, 45 by 39 μ; second cell, 40 by 37 μ, third cell, 39 by 35 μ; fourth cell, 37 by 33 μ. The oolemma is 10 μ in thickness. The polar bodies may be as large as 12 to 13 μ or as small as 8 to 10 μ.

The above data show that the unfertilized eggs, still sursounded by their corona radiata, are not spherical but slightly oval (fig. 2) at the pronuclear stage, as Huber has observed in fixed material. The oval form of the cell frequently becomes accentuated in bicellular and following stages. However, not all the eggs in the same tube have the same form (fig. 1) and dimensions, nor are all of them in the same cleavage stage. Sometimes eggs are discoidal in shape.

Starting from the bicellular stage (fig. 3), strictly spherical blastomeres are seldom observed. Usually their surface is rather irregular, frequently showing such protuberances as those represented in the wax models of Huber. Huber's deduction that the blastomeres, free from the compression caused in vivo by the tubal walls, should assume a spherical shape in liquid medium, is not strictly correct. Although the oolemma becomes oval in a liquid medium, and its surface smooth, the blastomeres remain irregular (fig. 4).

The cytoplasm seems to consist of a plastic but not elastic material, and does not react strictly according to the principles of surface. The irregular form of the blastomeres can be used as an index of the vitality and normal condition of the cytoplasm. If they are placed in an abnormal medium, as described later, they quickly assume a spherical or regularly oval shape. At the bicellular stage it is possible to distinguish in vivo one blastomere which is larger and more transparent than the other. It divides first, giving rise to the tricellular stage. At the tetracellular stage the second mitosis determines the appearance of two pairs of blastomeres which like the first two blastomeres differ in their volume (fig. 5). These observations are in accord with those of Lewis and Gregory ('29) upon living rabbit eggs.

The cytoplasm exhibits different aspects in the unfertilized eggs, in fertilized eggs at the pronuclear stage, and in the blastomeres. In the former, as Long has observed, the cytoplasm is optically heterogeneous and is not very transparent. The nucleus is concealed. Near the center of the cell two or three vacuoles can be detected. The oolemma, or zona pellucida, is clearly visible. A perivitelline space surrounds the cytoplasm and is particularly evident on the side of the first polar body. The eggs, before fertilization, are surrounded by a considerable number of follicular cells, and are held together by a very soft and sticky substance. In fertilized eggs the cytoplasm assumes a more transparent and homogeneous aspect. The vitelline granules are small and uniformly arranged, and mask the nucleus. Inclusions are not

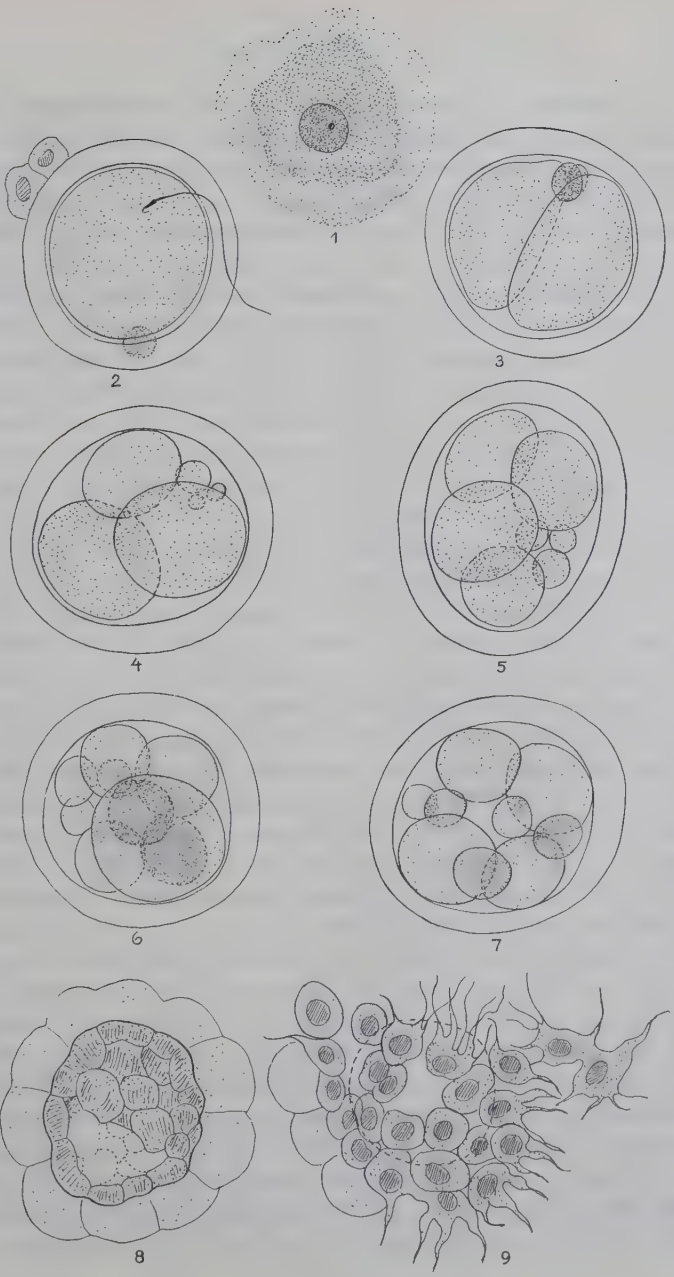
visible. After the first division the cytoplasm of the blastomeres becomes more transparent, perhaps because of the reduction of the cytoplasmic mass. In a limited zone of the cell, where the nucleus is probably located, there are some vacuoles (vacuome?). During segmentation the perivitelline space increases and usually it contains three polar bodies.

The eggs of the rat are extremely sensitive to variations in temperature, osmotic pressure and hydrogen ion concentration of the medium.[2] The lytic phenomena produced by experimental variations of the medium are: shrinking of the cytoplasm, intracellular precipitation with formation of dark spots separated by spaces optically empty, uniform clouding of the content, emission of hyaline droplets, swelling with dilatation of the oolemma so as to reach 100 to 120 μ in total diameter, immediate cessation of mitosis with a sudden disappearance of the cleavage furrow, and fusion of the blastomeres. At high (39°C.) and low (35°C.) temperature shrinkage occurs. Hypertonic solutions cause shrinkage and the expulsion of droplets from the cytoplasm, while hypotonic solutions cause swelling and then intracellular precipitation with shrinkage of the content toward the center. Hypotonic and hypertonic solutions do not modify the zona pellucida.

In acid medium below pH 7, and without protein buffers in the medium, the protoplasm in its entirety becomes more dense; in alkaline medium, above pH 7.8 to 8.0 the protoplasm becomes clear and transparent and swells, Definite cytoplasmic modifications were not detected when either the K or the Ca content of the solutions was increased or decreased.

The polar bodies are still more sensitive than the cytoplasm of the blastomeres. They cytolyze very easily and rapidly. The condition of the polar bodies, the irregular form of the blastomeres and failure of the nucleus to stain with neutral red are reliable criteria for judging the viability of the eggs and for controlling the physiological properties

[2] In this respect Doctor Defrise's material differs strikingly from Nicholas's results with older stages (7, 8, 9 days), which are not at all susceptible to rather large variations in these factors.



Figures 1 to 9

of the medium. The first stages of the degenerative phenomena mentioned above are reversible: the eggs can reassume their shape and original structure without dying.

In contrast to Huber's findings, the present observations indicate that rat eggs retain the oolemma or zona pellucida almost up to the time when they become fixed in the uterine crypt. The blastocyst loses the oolemma through a swelling and dissolving process, which gives to the external surface a mammellonated aspect (fig. 8). First the blastomeres and then the trophoblastic cells come into contact with the internal face of the zona pellucida, which gradually increases its own surface in so far as its elasticity will permit. The rat egg is similar to that of the rabbit as far as the behavior of the zona pellucida is concerned. It appears in fact from the observations on fixed material by Assheton (1896) and from those in vitro and in vivo by Lewis ('29) that the rabbit eggs lose the oolemma at the advanced blastocyst stage, very likely, as Lewis thinks, through a process of digestion by means of an enzyme secreted by the uterine mucosa.

The pathological eggs which were observed by Huber in the tubal and uterine lumina among eggs in the process of normal segmentation do not necessarily indicate that the retention of the oolemma is a causal factor in their degeneration. The presence of these abnormal eggs can rather be explained on the basis of observations recently made to determine whether or not there exists in mammals the possibility of parthenogenetic development.

The papers which treat of the segmentation of non-fertilized eggs in mammals can be divided into two groups. In one the possibility of parthenogenesis is affirmed; in the other it is denied. Branca ('25) observed, for example, in ovaries of rodents, not only parthenogenetic eggs at the bi- and tetracellular stage, but morulae and parthenogenetic blastulae with unicellular trophoblastic layer and a germinal knot. He also noted a case in which a cellular mass in layers, and a syncytium with large nuclei could be interpreted respectively as a primitive line and a 'placenta ovarica.' On the other hand,

Kampmeier ('30) denies a true parthenogenetic development in mammals because all the cases brought forth by supporters of parthenogenesis can be interpreted as being caused by degeneration of the ovular cytoplasm followed by follicular atresia. 'Cell balls' which at first might be interpreted as being derived from an ovarian segmentation, and which instead result from a particular type of atresia with invasion of follicular cells in the perivitelline space, were observed in human tubes by Allen ('30) and his collaborators.

Abnormal eggs in atypical segmentation (stages from six to sixteen blastomeres) surrounded by a normal oolemma were found in the genital tract (tube and uterus) among normal eggs in both virgin and fertilized females. The volume of these eggs and the transparency of the blastomeres—or of those at least which, from their appearance, could be called such—has always appeared normal. The only difference between these eggs and those whose segmentation is dependent upon the regular action of the spermatozoon is the marked dissimilarity of the relative volume of the blastomeres. If indeed it be true that cellular divisions have occurred they must have taken an entirely abnormal course. In figures 6 and 7 are illustrated two examples of such abnormal eggs, one found in a virgin female at the late oestrous period, the other in a fertilized female 48 hours after mating and among normal eggs at the bicellular stage. The volume of the cytoplasmic masses varies noticeably. The nucleus is invisible. According to Long and Evans from 625 deliveries (with a mean of 9.6 eggs per ovulation) the mean of the newborn was 6.9 per delivery. MacDowell's ('25) observations gave identical results. Long and Evans also found that one-third of the ovulations showed atretic follicles with retention of eggs. This means that one-third of all ovulations are accompanied by a retention of eggs in the follicle (the mean is uncertain for each case) and that in every ovulation one-third of the eggs do not produce foetuses. This mortality must include the abnormal eggs mentioned above and must be taken into consideration in the study of parthenogenesis in mammals, which

has so far been performed exclusively on follicular eggs. The results of the researches of Novak and Heisinger ('23) on tubal eggs showing the possibility of inducing artificial parthenogensis in rats have to be revised, in view of the constant presence of abnormal eggs in the tube.

Methods for securing eggs

The eggs are located, according to the period of development, either in the tubal or in the uterine cavity. Different techniques must be followed to secure material from the one or the other of the two cavities.

Tubal eggs. The impossibility of washing the tube in situ because of its very narrow lumen and twisted convolutions requires that salpingectomy be practiced.

The tube is removed and placed in Tyrode solution or blood serum in a Syracuse watch-glass lined with black paraffin. It is fastened with pins through the whole thickness of the uterine stump, and under a dissecting microscope, kept in a thermostat, the mesosalpinx is removed by small iridectomy scissors. Free from its own peritoneal ligament the tube is extended on the paraffin by means of a pin through its ovarian extremity.

In the case of unfertilized eggs (female at stage IV) the eggs are obtained through a small incision in the tubal wall, which appears swollen and transparent. As soon as the incision is made a mucilaginous transparent cloud emerges, containing the eggs surrounded by their own corona radiata (fig. 1). These can be easily removed with a pipette and placed on a depression slide.

In the case of fertilized eggs the entire length of the tube is cut longitudinally and the eggs removed. It was found that very often under the stimulus of the incision the tubal peristalsis would push all the eggs into the vicinity of the uterine ostium in a single mucilaginous mass which could be picked up by touching the end of a pipette against the uterine mucosa of the extreme distal part of the horn.

Uterine eggs. Eggs from the uterine cavity can be secured in situ. By means of a fine needle mounted on a syringe with a tightly fitted piston, from 0.5 to 1.0 cc. of the liquid to be used is injected into the uterine cavity. The liquid is then withdrawn and placed on a depression slide. From the depression slides single eggs can be easily withdrawn in a pipette with an open end of from 0.5 to 0.7 mm. and, if necessary, may be washed in various liquids.

Culture of eggs

The rat eggs, unlike those of the rabbit which segment in vitro when they are cultivated in homologous plasma (W. Lewis and Gregory, '29) do not segment regularly in vitro when they are cultivated in the following solutions: 1) Ringer's solution, buffered or not with sodium bicarbonate; 2) Tyrode's solution, A) isotonic with NaCl, milimol: a) 120, b) 136, c) 151; B) $K^+ - Ca^{++} - Mg^{++}$ equilibrium on the basis of the triangular diagram of Löewe; milimol: a) 2.65 KCl, 1.90 $CaCl_2$, 2.20 $MgCl_2$: b) 5.63 KCl, 2.16 $CaCl_2$, 0.52 $MgCl_2$: c) 2.65 KCl, 3.60 $CaCl_2$, 0.52 $MgCl_2$: the above solutions were used at pH 6.8, 7.2, 7.6. 3) Tyrode's solution (NaCL: milimol 136 – KCl: 5.6 – $CaCl_2$: 2.16 – $MgCl_2$; 0.52 $NaHCO_3$: 8.6 – $C_6H_{12}O_6$: 5.5) with the addition of gelatine 0.5 per cent. 4) Tyrode's solution (as above) with the addition of blood serum: a) 1/1, b) 3/1. 5) Tyrode's solution(as above) with the addition of plasma (heparin): a) 1/1, b) 3/1. 6) Pure blood serum of a) pregnant female, b) male, c) newborn. 7) Plasma (secured from the heart and mixed with heparin): a) pregnant female, b) male. 8) Spinal fluid (secured by suboccipital puncture). 9) Foetal hystolymph (secured by Martinovitch's ('29) technique). 10) Uterine fluid (II oestral period): a) pure, b) with the addition of blood serum.

In a few cases, in some of the above media, and especially in this: NaCl milimol 130 – KCl 2.65 – $CaCl_2$ 174 – MgCl 1.18 – $NaHCO_3$ 8.6 – at pH 7.2 – drops III + blood-serum drops II, one or two mitoses were obtained. The addition of small quantities of embryonic extract, of rat folliculine, of extract

of the anterior lobe of the hypophysis to the medium (either solid or liquid, natural or artificial) has not noticeably modified the culture results.

The liquid which is collected in the uterus during the oestrus, and which seems to represent the normal physiological medium for the spermatozoa during their passage in the uterine cavity is not suitable for the segmentation of the eggs.

Cultures of the blastula

The volume of the early blastula does not differ much from that of the eggs. First they are spherical and surrounded by a clearly visible ooelemma. When the oolemma begins to swell they present an external furrow corresponding to the limit of the roof (fig. 8). In vivo, during the initial stages, the cavity occupies about half the total volume and it is limited in its inferior portion (trophoblastic) by cuboidal cells regularly arranged, with well-marked limits, which in vital staining by neutral red present numerous granules in the cytoplasm bordering the blastocoele. The nuclei of the cells are easily discernible.

Cultures of initial blastulas in plasma + Tyrode's solution, especially when the drop spreads and the blastula comes in contact with the surface of the cover glass, show an architectonic disorder of the blastula, cellular migration, branching cytoplasm, protoplasmic bridges (fig. 9). After 48 hours of culture the cells apparently do not differ from a fibroblast-like cell cultured in vitro for a certain time.

DISCUSSION

The cellular changes which occur in the in vitro culture must be regarded as degenerative phenomena. These changes are not the same for the stages of development here studied. The segmenting eggs are not capable of dedifferentiation. The older stages are capable of undergoing dedifferentiation to a slight degree.

In this respect the different stages of rat development are comparable in their reaction to other types of cells which

have been observed in tissue culture. The blastomeres, incapable of dedifferentiation, react as do adult nerve cells in unusual or abnormal media. They exhaust their own 'residual energy' and die.

The blastulae when cultivated in vitro undergo some degree of dedifferentiation which tends toward a leveling of their differential cellular characters. Their individual components give rise to a fibroblast-like cell which probably is a particular type developing only under abnormal conditions.

SUMMARY

1. Living unfertilized rat eggs are not spherical but are oval in form as observed by Huber ('15) in fixed material.

2. Eggs of different cleavage stages and varying forms and dimensions are found in the same tube.

3. The irregularity of the blastomeres is an index of the viability of the eggs. Regularity (spherical or oval form) presages abnormality and death.

4. The cytoplasm of the unfertilized egg is optically heterogeneous as observed by Long ('12). After fertilization it becomes much more homogeneous.

5. Attempts to culture rat eggs showed that they are exceedingly sensitive to small variations in temperature, osmotic pressure, and hydrogen ion concentration.

6. The rat eggs survived for a limited length of time in the media used but did not undergo any degree of development. Cleavage was obtained in several cases but was limited to one or two cell divisions.

7. Cultures of blastulae were maintained for 48 hours, at which time the cells began to lose their character and become fibroblast-like in nature.

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A STUDY OF THE NUMBER AND LOCATION OF THE PARATHYROID GLANDS IN MAN

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THREE PLATES (TWENTY-SIX FIGURES)

Even though the greatest care is taken is operating on the thyroid gland, to avoid injury to the parathyroids and to preserve their blood supply, the surgeon is still handicapped by the inadequacy of our knowledge of their precise location and their variation in number.

There have been several studies of the number and the positions of the parathyroids in man, but the grand total of cases accurately recorded, so far as I have been able to collect them from the literature (381), is comparatively small. It seemed, therefore, that a careful study of additional cases, treated from a standpoint which would be of use to the surgeon, would be of value.

PROCEDURE

The material used in this study consisted of twenty-five human specimens of which thirteen were obtained from the dissecting room of the University of Pennsylvania, and twelve from autopsies at the Philadelphia General Hospital.

The thyroid glands and neighboring structures were dissected carefully, and when suspected parathyroids were found parts of them were embedded in paraffin, sectioned, and examined microscopically.

[1] This paper includes material collected in partial fulfillment of the requirements for the master of science degree at the University of Pennsylvania, and filed as a thesis in the University of Pennsylvania library.

It cannot be claimed that every trace of parathyroid tissue, present in each specimen, was discovered. In dealing with structures as small and as variable in location as the parathyroids, one could only be certain of making a complete study by embedding and sectioning the entire thyroid glands and all the tissue in their neighborhood, including the retrosternal tissue, a procedure which would be so time consuming as to make it prohibitive.

From the ninety-seven specimens of suspected parathyroid tissue, eighty-six pieces were found microscopically to be parathyroid glands. In most cases the discrepancy was caused by suspecting small lobules of fat and accessory thyroids.

Frequently the parathyroids were encased in fat, making identification with the naked eye somewhat doubtful; however, with this exception there is little difficulty in macroscopic recognition. Even though there are many bodies of similar location and size, the pinkish-brown, translucent, smooth appearance of the lenticular, flattened, sharp-edged bodies is so characteristic that, in the majority of the cases, they can be distinguished readily from the red, coarsely granular appearance of the accessory thyroids, which are generally attached to the thyroid gland proper, and from the rounded firm, grayish-pink to black colored lymph nodes, and yellowish non-encapsulated lobules of fat.

Precise natural size drawings were made of the thyroid glands in each case, and the positions of the parathyroids were plotted in relation to them, on the bases of accurate measurements. Likewise, a composite drawing was constructed from all the parathyroids found in twenty-five cases, plotted on a thyroid gland of average size and shape.

NUMBER OF PARATHYROIDS

There is a wide variation in the number of parathyroids found in different specimens. In the twenty-five cases studied, the number varied from two to six per person, as shown in table 1; an average of 3.2 parathyroids for each individual.

TABLE 1

Number parathyroids	*Number of cases*	*Percentage*
2	5	20
3	10	40
4	6	24
5	2	8
6	2	8

A comparison of the number of parathyroid glands per specimen found by the author, with the findings of other investigators, is shown in table 2:

TABLE 2

	Number cases	*Average number per case*
Berkeley ('09)	125	2.5
Pool ('16)	16	2.9
Geis ('18)	12	3.0
MacCallum ('16)	64	3.2
The author ('31)	25	3.2
Yanase ('28)	89	3.3

LOCATION

From the specimens studied, the following are the observations regarding the location of the parathyroid glands.

In general, the parathyroids lie most frequently upon the medial half of the dorsal surface of the lateral lobes of the thyroid, between the common carotid artery and the inferior constrictor muscle of the pharynx above, and the oesophagus below, lateral and dorsal to the recurrent (laryngeal) nerve but not in intimate relationship with it.

In relation to the capsule of the thyroid gland, they are most often found in the loose areolar tissue just outside it, less frequently adherent to its outer surface, and still more rarely within its split layers. In no instance did I find a parathyroid adherent to the thyroid gland, nor of the eight thyroids grossly sectioned, could I find any trace of the parathyroids within the substance of the thyroids. In autopsy cases where the tissue was comparatively fresh, the parathyroids could be lifted with the surrounding tissue, well away from the surface of the thyroid gland, without disturbing the thyroid.

Regarding the specific location in individual cases, the variation is so great that it would not be inaccurate to state that no two specimens were alike. This is brought out by a study of the individual pictures, figures 1 to 25, and still more strikingly by a glance at the composite drawing, figure 26.

Among the eighty-six glands studied, seventy-six were located dorsal to the thyroid gland, one at the ventro-caudal pole and nine caudal to it. Of the latter, four were in contact at one end with the thyroid capsule, and five were entirely away from it, lying in the loose tissue ventral and lateral to the trachea, or in a cord of tissue extending from the thyroid capsule to the thymus.

Of the seventy-six glands found dorsal to the thyroid, six were in relation with the cranial third while the remaining seventy were distributed fairly evenly in relation with the caudal two-thirds of the gland, except for a rather noticeable concentration at the region of the cricoid cartilage and the first tracheal ring. Nearly all were in relation with the dorsal surface of the thyroid, the main exceptions having one located between the isthmus and the trachea, three in the triangular cleft formed by the thyroid gland, trachea, and oesophagus, and one nearly in the mid-line between oesophagus and trachea.

In addition to the definite parathyroid glands included in the total described, there were found in sections a few non-encapsulated masses of what appeared to be parathyroid tissue surrounded by fat.

DISCUSSION

The various locations in which parathyroids were found in this study are paralleled by the findings of previous investigators. In addition, parathyroids have been described in still other locations (in which I found no representatives) as follows: within the thorax they have been described as embedded in thymic tissue, in the posterior mediastinum, and in the areolar tissue about the pericardium. Verebely states that small portions of the parathyroids may appear in the

thyroid, and also that the thymic tissue may be present in the normal parathyroid.

Pool mentions the finding of a large parathyroid on the anterior surface of the isthmus of the thyroid gland in one case. Millzner found in forty-two cases, fifteen pairs of parathyroids, 35.9 per cent on the ventro-lateral thyroid capsule and of these, 9.5 per cent lay on the true ventral capsule. These parathyroids were described as being typical in size and relation to the thyroid vessels and capsules, and appear to represent true ventral displacements. Willbrock ('29) found one or more pairs on the ventro-lateral surface, in approximately 8 per cent of 1056 thyroids examined.

Accessory parathyroids, that is small masses of typical parathyroid cells without a definite capsule, have been described by Muller and Erdheim, and seem to have no definite location or number.

As stated earlier, one can never be certain that he has found all the parathyroids in any specimen by gross dissection alone, and it would be misleading to leave the impression that the number of parathyroids found per specimen by the various investigators, including the author's, represents the total number present. While the average number is fairly uniform, for the different authors, there is always the possibility that one or more glands have been overlooked. However, until proven otherwise, the possibility must certainly be kept in mind that there may be occasional cases in which only one parathyroid is present, others with not more than two, and perhaps a larger number with not more than three.

It is interesting that none of the investigators found four glands in much more than 50 per cent of the specimens studied, while an appreciable percentage of specimens was found to possess five or six glands apiece, and one investigator found as many as eight parathyroids in a single specimen.

Most anatomy texts describe as 'typical,' two pairs of glands with a definite, 'typical' location for each pair. In this study, four glands were found in each case in only 25 per cent of the cases studied, and the positions of the glands in

but two of these cases corresponded to the textbook description of the 'typical' location.

It would, therefore, seem to be more in accordance with known facts, in describing the parathyroids, if textbook writers would emphasize the wide variation in both number and position, instead of making a dogmatic statement which applies to considerably less than the half the cases, and which is not only inaccurate but dangerously misleading.

SUMMARY

The parathyroids were studied by gross dissection supplemented by microscopic inspection in twenty-five human specimens. A total of eighty-six definite parathyroid glands was found, making an average of 3.2 per specimen.

The number varied from two to six. The location of each parathyroid was determined and reported in relation to the thyroid, larynx, and trachea. An analysis was made of the variations in location which showed that 7 per cent of the glands were opposite the cranial third of the thyroid, 57 per cent the middle third, and 25.6 per cent the caudal third, 10.4 were found caudal to the thyroid. One gland was found partly on the ventral surface; none in the interior of the thyroid.

It is suggested that the customary nomenclature, according to which, "there are two pairs of parathyroids, superior and inferior," with a typical location for each pair, is misleading, since no investigator, who has carefully studied and analyzed a series of cases, has found exactly four parathyroids in much more than 50 per cent of the specimens, and since in many cases where four have been found, there is a great variation in location.

This study was made possible by the kind guidance and aid of Dr. Eliot R. Clark, Professor of Anatomy, University of Pennsylvania Medical School.

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EXPLANATION OF ALL PLATES

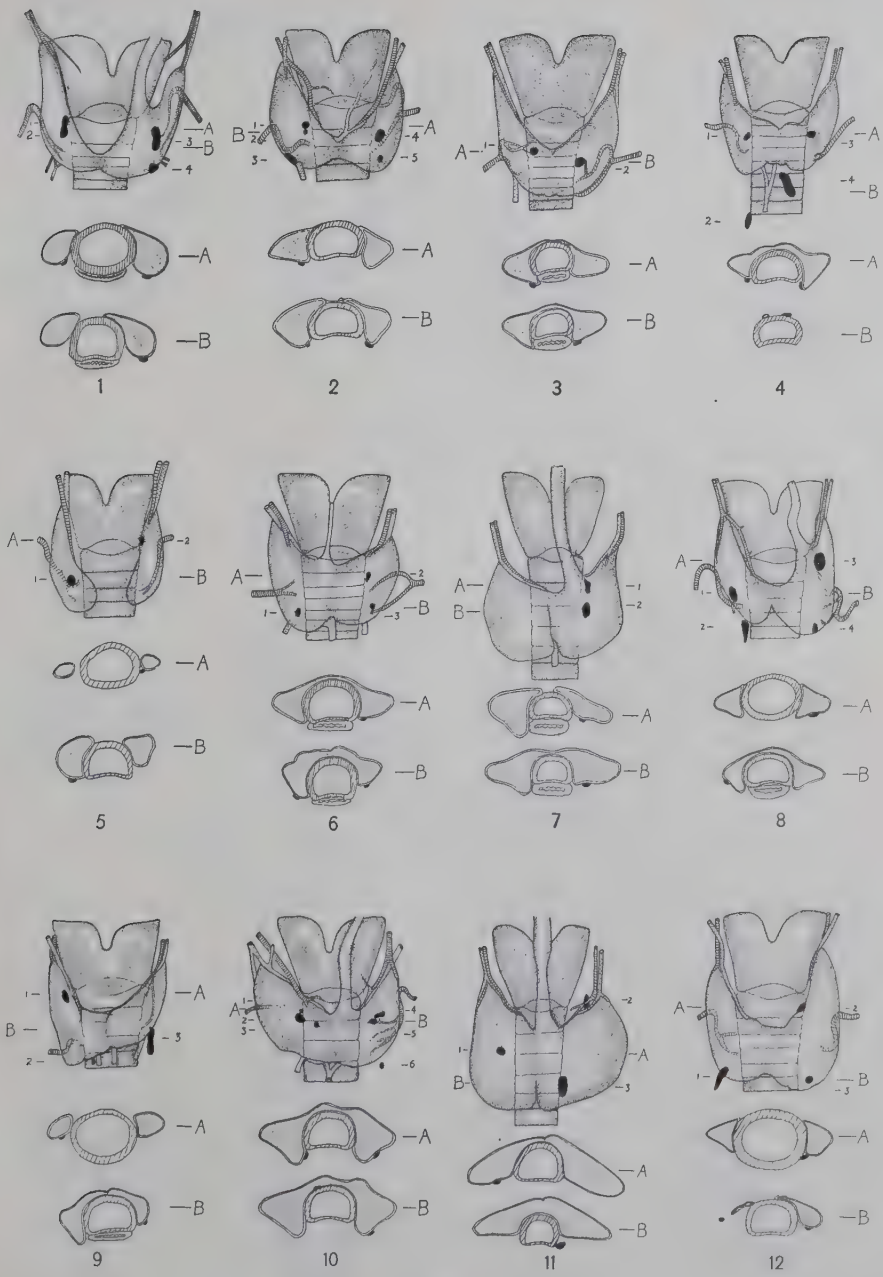
Figures 1 to 25 are reproductions of drawings of the thyroid and the parathyroid glands and neighboring structures.

Figures 1 to 12 and figure 25 are from dissecting room material; the locations of the glands are shown in cross section in addition to the ventral view.

Figures 13 to 24 are from autopsy material; both the ventral and the dorsal views are given. In the ventral views the parathyroids, which are dorsal to the thyroid, are drawn as if the latter were transparent. The various parathyroid glands in each case are numbered from one to six.

Figure 26. This diagram represents a composite of all the parathyroids found in the twenty-five cases, plotted on a thyroid gland of average size and shape. The upper brackets indicate approximately the upper, middle, and lower thirds of the thyroid; the lower bracket the space below the thyroid gland; the numbers giving the counts of the parathyroids within each bracket. The parathyroids are represented as if the thyroid were transparent.

An attempt has been made to indicate the parathyroids which might be called 'superior parathyroids' in outline and 'inferior parathyroids' in black. Those glands which could not be classified either by location or by being one of a pair, are cross-hatched.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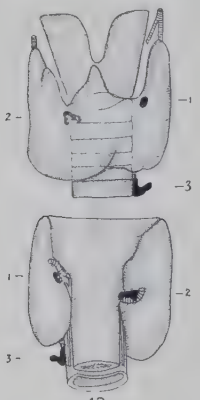
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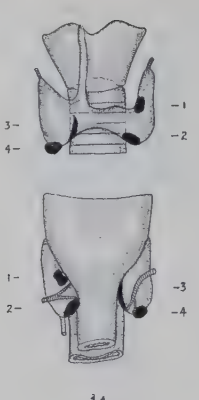
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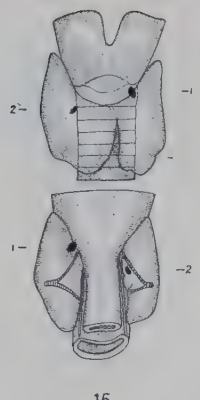
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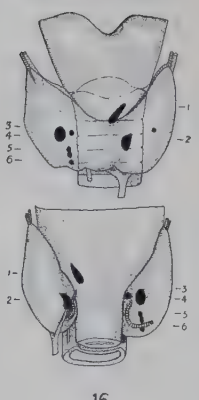
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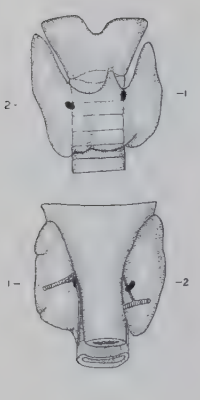
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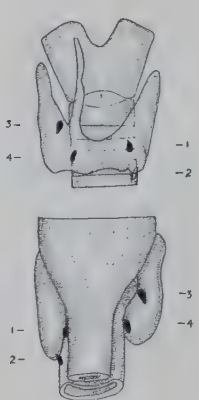
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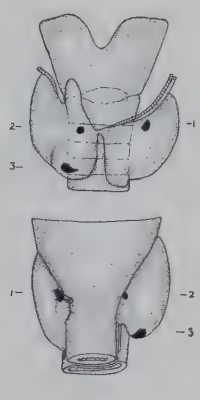
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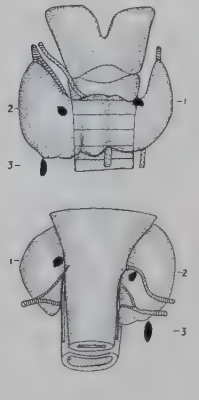
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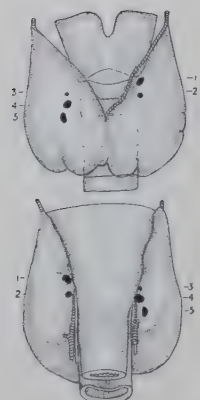
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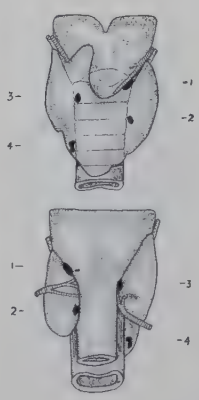
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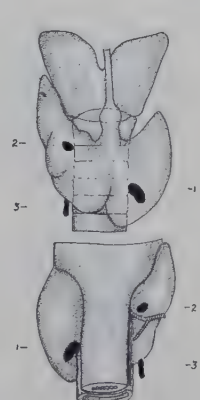
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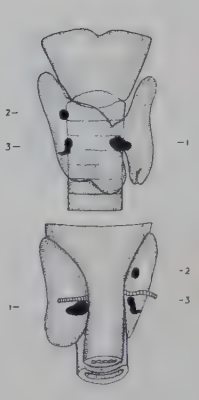
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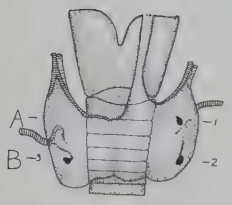

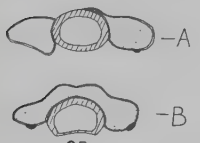

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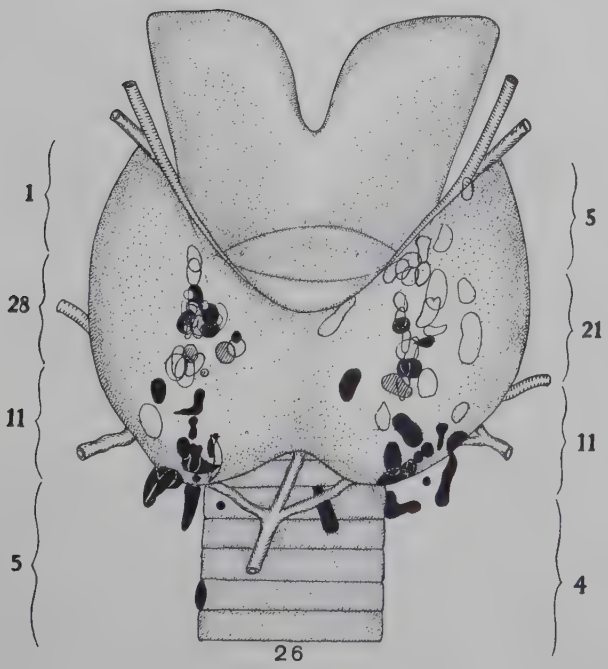

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STUDIES ON THE LYMPHATIC VESSELS AND ON THE MOVEMENT OF LYMPH IN THE EAR OF THE RABBIT [1]

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FOUR FIGURES

Studies made in this laboratory on lymphatic vessels, as seen in transparent chambers introduced into the rabbit's ear (Clark and Clark, '30–'31, '31, '32 a, b, c) have brought out a number of new facts regarding the mode of growth of lymphatics, and the activity of the newly formed lymphatics. Since in all of these studies only the lymphatics seen in the chambers were involved, it seemed desirable to investigate the lymphatics of the normal ear—their distribution, relations, and activity, and to attempt to find out the extent to which they are modified by the introduction of the artificial chambers. This paper presents the results, some of which are given as preliminary reports.

It became apparent, soon after the work was started, that the normal ear of the rabbit offers a very favorable field in which to extend our knowledge of the behavior of lymphatics.

METHODS

The methods used included single and double injections of the lymphatics and blood vessels, injections of lymphatics, and observations of the movement of the injected granules in the living animal, the collection of lymph from the larger vessels of the intact ear, and the introduction of transparent chambers into the ear.

[1] The work in this laboratory on the study of living cells and tissues in the transparent chamber inserted in the rabbit's ear is being aided by a 5-year grant from the Rockefeller Foundation.

Injections of the lymphatics were made beneath the epidermis with an ordinary hypodermic syringe and a 30 gauge steel needle. In the intact ear, the substances injected were 50 per cent India ink in tap water, a saturated solution of Berlin blue in distilled water, and a saturated solution of methylene blue in 0.9 per cent sodium chloride solution. The specimens injected with India ink and Berlin blue were fixed in 10 per cent formalin, dehydrated in alcohol, and cleared in oil of wintergreen.

The methylene blue injections were made by the supravital technique as described by R. G. Williams (Bazett et al., '32) and as applied by him to the rabbit's ear (R. G. Williams, '31). They were carried out at Doctor Williams' suggestion, and their success was dependent upon his assistance and advice.

Blood vessels were injected with Ranvier's carmine-gelatin mass (Lee) immediately after killing the animal with chloroform and washing the vessels with Ringer's solution. In double injections, the lymphatic vessels were injected while the animal was alive. Then the blood vessels were injected as described.

In collecting lymph, the animal was placed on its back or side, the ear extended horizontally, or with its tip slightly dependent. The anaesthetic used was: 1) nerve block with 1 per cent cocaine solution; or, 2) nerve block with 2 per cent procaine solution; or 3) sodium amytal intraperitoneally or intravenously, 300 to 500 mg., depending upon the size of the rabbit and the duration of the experiment.

The cannulae used for collecting lymph were drawn out from ordinary glass tubing. They were calibrated in millimeters to facilitate observations. Their inside diameters (average, 1 mm.) were determined by an ocular micrometer in order to calculate the volume of lymph. Working under a binocular microscope, a transverse incision was made near the junction of the proximal and middle thirds of the ear, over a large blood vessel, preferably the median (main) artery. An accompanying lymphatic was isolated, distended

with lymph by massage toward that point, and a cannula introduced. An attempt to limit the flow of lymph in anastomosing vessels was made by adding a weight proximal to the site of operation—a weight heavy enough for this purpose, but light enough to have practically no effect upon the flow of blood.

The type of transparent chamber used was the 'preformed tissue' chamber (Clark et al., '30). In its insertion, the ventral (inner) skin and the cartilage of a portion of the ear are removed with a minimum of injury, the mica cover of the chamber being brought into contact with the subcutaneous tissue of the dorsal (outer) surface. Thus, the tissue and vessels observed are those already present ('preformed') in the ear at the time of operation. In the insertion of this type of chamber, a kodaloid disc or 'platform' 0.62 mm. thick, is used which occupies that part of the chamber away from the median artery, nerve, and lymphatic collecting vessels. The use of the 'platform' produces two distinct regions, namely, a thinner region over the disc, which will be called the 'platform' region, and a thicker region in which the large vessels and nerves are located, which will be called the 'groove.'

Injections used in conjunction with chambers were 33 per cent India ink in sterile tap water, sterile 1 per cent carmine in 3 per cent gelatin, and a sterile saturated solution of Berlin blue in 3 per cent gelatin.

THT DISTRIBUTION OF LYMPHATICS

Specimens of the intact ear, injected with India ink, show two rich lymphatic plexuses, deep and superficial, on each surface (fig. 2). Around the periphery, these are composed largely of numerous small capillaries with blunt ends. Nearer the center, the superficial layer contains many capillaries similar to those of the periphery. The deep layer, situated near the cartilage is composed of relatively large vessels, containing numerous anastomoses. These lymphatics empty into slightly larger collecting vessels, which for the most part follow the distribution of the larger blood vessels, form-

ing networks around them (fig. 1). Like the blood vessels (Hoyer, 1877), the lymphatics of the ventral surface penetrate the cartilage in many places, and empty into the collecting vessels on the dorsal surface (fig. 2).

In the general convergence of vessels at the base of the ear, the number of larger collecting lymphatics exceeds that of the large arteries and veins. Usually several lymphatics accompany each of these blood vessels. The total number of collecting lymphaticse at the level at which the cannulae were

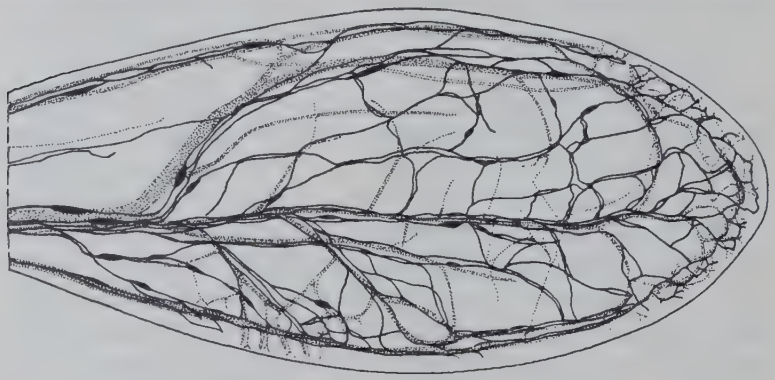

Fig. 1 General distribution of collecting lymphatic vessels (represented by solid black) of the rabbit's ear compared with that of the larger blood vessels (represented by dots). This illustration is a composite drawing made from a number of cleared specimens in which the lymphatics had been injected with India ink, and the blood vessels with carmine-gelatin mass. Natural size.

inserted varies from 10 to 14. In their further course, there is very little reduction in number, 8 to 12 running separately into the auricular nodes.

The lymphatic vessels of the rabbit's ear are endothelial tubes around the larger of which are small and varying amounts of connective tissue. In the specimens injected with methylene blue, occasional smooth muscle cells were seen on some of the larger lymphatics, but they were very few and irregularly spaced (fig. 3). The paucity of extra endothelial tissue is such as to indicate the likelihood that substances might diffuse readily into practically all of the

lymphatics of the ear, including even the larger vessels. Whether this does occur normally, however, has not been tested.

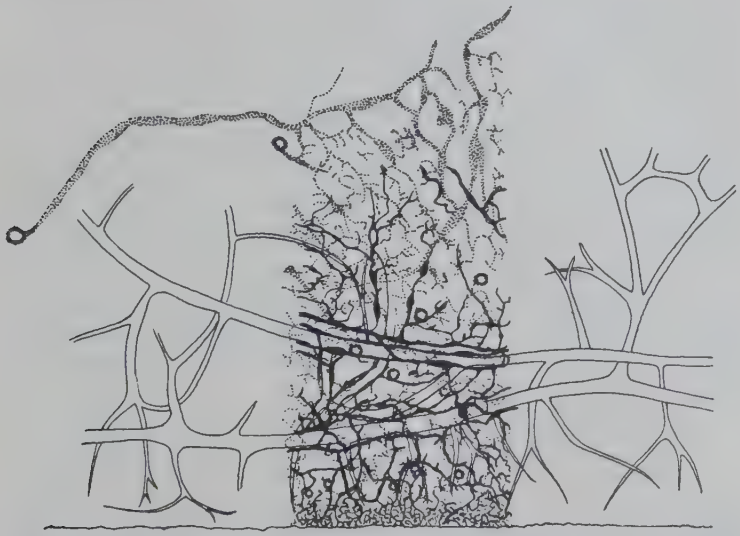

Fig. 2 Lymphatic capillaries at the periphery of the rabbit's ear. A number of lymphatics may be seen going through the cartilage (represented by circles). The blood vessels are shown in outline. Magnification, × 10. Lymphatics of dorsal side dotted; lymphatics of ventral side solid black.

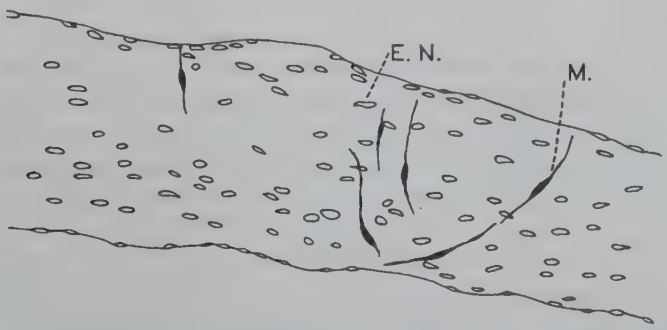

Fig. 3 The appearance of a large lymphatic collecting vessel in a rabbit's ear injected supravitally with methylene blue, showing endothelial nuclei (E.N.) and smooth muscle cells (M.). Magnification, × 130.

THE AREA OF LYMPHATICS

The relative surface area of lymphatics and blood vessels was determined upon injected specimens. The inaccuracies of this method are appreciable and the results are presented for their comparative rather than actual values.

The blood vessels in six fields and the lymphatics in another six fields were studied. Each field was selected for the completeness of the injection and was 0.75 mm. square. Drawings were made with a camera lucida at a magnification of 160. The areas (projections of the greatest dimensions of the vessels as seen from the surface) were computed with a planimeter. The average of these was taken, and reduced to actual size. From these results, determinations were made for 1 sq.mm. of surface area of ear. Assuming the vessels to be cylindrical, these figures multiplied by π give their surface areas. The results are:

Surface area of lymphatic vessels, 1.43 sq.mm. per sq.mm. surface of ear
Surface area of blood vessels, 1.63 sq.mm. per sq.mm. surface of ear

Calculations on living vessels were made in a similar manner in a 'preformed tissue' chamber. In this case only the vessels of the dorsal surface were measured, and only two fields of each were taken. The lymphatics were injected with India ink. The blood vessels contained an average amount of blood. The results are:

Surface area of lymphatic vessels, 1.36 sq.mm. per sq.mm. dorsal surface of ear
Surface area of blood vessels, 1.18 sq.mm. per sq.mm. dorsal surface of ear

From the results obtained from measuring several ears, 5000 sq.mm. (nearly 8 sq.in.) may be taken as the average area for one surface. For such an ear, the total surface area of lymphatics would be 5000×1.43 or 7150 sq.mm.

THE MOVEMENT OF LYMPH

The movement of injected particles (India ink, carmine, Berlin blue) in the smaller lymphatics of the intact ear occurs in both directions. In the larger vessels it is limited to proximal flow by numerous competent valves. Retrograde

injection, after the insertion of cannulae was found impossible. Similarly, once injected material enters the larger vessels, there is little or no return to the smaller.

With little force from behind, injected particles dart rapidly along. The many anastomoses permit particles injected at one site to follow a number of routes. Material injected on the ventral side of the cartilage readily runs through to the other. This is particularly noticeable after the injection of India ink on one side and Berlin blue on the other. Although the black ink is dominant, the appearance of contrasting colors in confluent vessels is sometimes quite striking.

Even when the larger vessels are ligated proximally, only slight pressure is necessary to carry particles injected in the smaller vessels into them. They become irregularly distended due to fusiform supravalvular dilatations. These occasionally rupture, even after injection has been completed. Very soon after the injection is stopped, rapid movement of the particles ceases. They are then seen to move along when helped by massage or by active movement of the ear. There is seen also an intermittent movement, apparently spontaneous.

In the 'preformed tissue' chamber, the following factors have been observed to produce flow of lymph, as indicated by the movement of particles in it:

1. Changes secondary to blood vascular phenomena; a) A slight back and forth movement, synchronous with arterial pulsation, and apparently caused by its transmitted force (compare Clark and Clark, '32 b). b) A longer excursion due to changes in caliber of the blood vessels. Thus, when the median artery contracts, particles in lymphatics in the 'platform' region of the chamber move toward it, and when it dilates, they move away from it (compare Clark and Clark, '32 b).

2. Active movements of the ear by the animal.

3. Massage. This produces rapid movement toward collecting vessels and along them centripetally.

4. Force from behind, as in injections.

5. The position of the ear. Movement is sometimes caused when the ear is held vertically, but the effect produced in this way has always appeared small.

Immediately after the insertion of a 'preformed tissue' chamber, some lymphatics are seen filled with masses of blood. Several days later these masses begin to move back and forth through short excursions, apparently caused by the transmitted force of arterial pulsation. They begon to break up, and after a number of days, the blood moves on. Then the lymphatic vessels appear as long, narrow, clear spaces, due to the colorless lymph contained. They vary in width from day to day. A lymphatic which yesterday was prominent may be invisible today, and one whose existence was unknown may appear. Injections show that relatively few of the total number are visible at any one time. It should be emphasized that this applies only to the 'preformed tissue' chamber, and not to the much thinner 'round-table' chamber, where all lymphatics are visible at all times (Clark and Clark, '32 b, '33).

Ten to fourteen days after the insertion of a chamber when recovery seems complete, particles of debris are occasionally observed in the small lymphatics of the 'platform' region. They may remain there for varying lengths of time—a few seconds to several days. That they are free is shown by their to and fro motion, and by their ready movement when the mica cover of the chamber is depressed, or when massage of the tip of the ear produces a rapid current of lymph.

While injected particles, once having reached the larger vessels accompanying the median artery, tend to move proximally, not infrequently some move peripherally into the vessels in the 'platform' region of the chamber. Thus, material inserted at the tip of the ear has been seen to appear in the chamber, 5 cm. away. Under ordinary conditions, following the injection of as much as 0.1 cc. this appearance is almost instantaneous. In small injections and in inflamed conditions the appearance is delayed.

Little or no obstruction is offered to the flow of blood by the 'preformed tissue' chamber. But there seems to be in the 'platform' area pressure sufficient to cut off lymphatic communication peripherally. With one exception, all material, no matter how close to the chamber it was injected, flowed around and entered the chamber via the collecting vessels in the 'groove,' accompanying the median artery.

OPENINGS IN LYMPHATICS

In the first few days after the insertion of a 'preformed tissue' chamber, places have been observed where fluid and erythrocytes seemed to come out from the lymphatic vessels to the fluid layer present between the surface of the tissue and the mica cover. In the same places, flow in the reverse direction, also, was observed. At times distinct openings were discerned (compare Clark and Clark, '33).

In order to make certain that these were actual openings between the lymphatics and the extravascular space, India ink, carmine, or Berlin blue was injected into the lymphatics distal to the chamber. The granules so injected passed along lymphatics to the chamber area, and in a number of cases streamed out into the layer of fluid over the top of the tissue through small but definite openings. The number of such was always small, varying from two to four per specimen.

A typical study of such openings, with the aid of injected granules in one specimen is as follows:

Three days after the insertion of the chamber, erythrocytes were seen emerging from and re-entering a lymphatic through an opening at the base of the 'platform' area, near the 'groove' (designated opening 1; see fig. 4). The next day this opening was obscured by extravasated erythrocytes between the tissue and mica cover.

Eight days after the insertion of the chamber, these erythrocytes had largely disappeared. India ink was then injected near the tip of the ear. The ink granules were at once seen passing through collecting lymphatics in the 'groove.' Some entered lymphatics on the 'platform' of the chamber,

and from one of these vessels, particles could be seen emerging and becoming free in the fluid space between the tissue and the mica cover. With the compound microscope (magnification of 100) the course of the lymphatic and the opening itself could be distinctly seen (designated opening 2). On this day, no particles were seen re-entering. The movements of the particles within the lymphatic vessels were of the

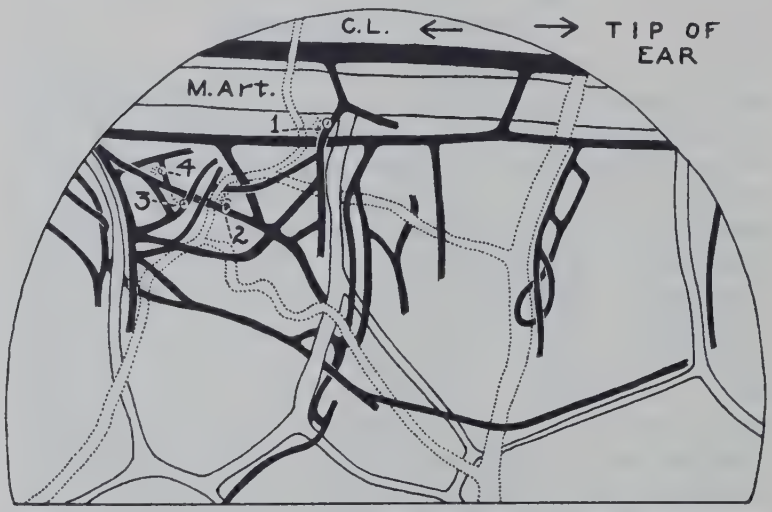

Fig. 4 Sketch of a part of 'preformed tissue' chamber (3-9-32L) illustrating the location and appearance of the four collections of India ink granules outside of the lymphatic vessels. Made on the ninth day after the installation of chamber, and 24 hours after the injection of India ink into the tip of the ear, distal to the chamber. The numbers in the figure corresponded to the designations given the sites in the text. Magnification. × 7.

kinds already mentioned as due to forces produced by changes in the blood vessels (v. s.), but movement forward exceeded that backward. Very gentle massage produced a rapid movement forward, and an outburst of particles from the opening.

On the ninth day, four collections of carbon granules were seen. Two were around openings 1 and 2. At the other two, no openings were seen. An occasional erythrocyte or ink granule came from opening 2.

On the following day, the collections of granules were unchanged. Erythrocytes were emerging and a few were reentering at opening 2. While watching one of the collections of granules at which no opening had been seen, erythrocytes appeared and a small opening was identified (opening 3). At the fourth site, erythrocytes appeared at a definite location, but no distinct opening was identified.

On the eleventh day, an occasional erythrocyte came out or went in through opening 2. None appeared at the other sites. Massage of the tip of the ear increased the flow from opening 2, but produced no perceptible effect at the other sites. During the next 2 days, the condition remained unchanged.

On the thirteenth day, an injection of carmine was made beside the previous injection of ink. Carmine granules appeared at and emerged from opening 2 in about 30 seconds. Other granules were seen traversing uneventfully the vessels formerly containing openings 3 and 4, passing the sites as through normal vessels, and none emerging.

The following morning, many more granules were around opening 2, concealing it. A few were around opening 1. None were at or near sites 3 or 4. On the 5 following days, no change was noted, and nothing was seen emerging or entering at 1 or 2. India ink was injected again. Particles passed uneventfully through all the lymphatics formerly containing openings, none emerging.

Thus, three distinct openings, whose existence was confirmed or discovered by the injection of granules into the lymphatics, were watched from day to day. One persisted for 10 days, and two for 13 days after the insertion of the chamber.

In a later chamber, two similar openings were observed from the second through the eighth day.

THE AMOUNT OF LYMPH

The results of the collection of lymph by the introduction of cannulae into the larger vessels are given in table 1.

When the collection was begun, the temperature of the ear was usually just a little above room temperature, and varied upon different occasions from 25°C. to 29°C. After an hour or so, the ear was heated by electric lights to 38°C., and the temperature maintained at that point for as long as an hour. This increase in temperature was followed by dilatation of the blood vessels, and a speeding up of the flow of blood. But no increase in the rate or amount of flow of lymph was noted.

Since the lymph collected was in each case from only one of the eight to twelve parallel vessels draining the ear, it was but a fraction of the total lymph flowing from the ear.

TABLE 1

The collection of lymph by cannulae introduced into lymphatic vessels of the rabbit's ear

EXPERIMENT	ANAESTHETIC	SPONTANEOUS FLOW	TIME	MASSAGE FLOW	TIME	TOTAL FLOW	TOTAL TIME
		Cu.mm.		*Cu.mm.*		*Cu.mm.*	
1	Cocaine	9.75	4° 15′	69.81	25′	79.56	4° 40′
2	Cocaine	15.07	2° 15′	1.94	1′	17.01	2° 16′
3	Sodium amytal	c.2.00	1°	0.50	10″	c.2.50	1° 10″
4	Sodium amytal	0	2°	25.57	3°	25.57	5°
5	Sodium amytal	44.0	4° 55′	19.50	20′	63.50	5° 15′

This fraction is variable, and its value can be only roughly estimated. From the effect of massage in various parts of the ear while the lymph was being collected, and from injections made at the completion into different parts of the ear to determine the area drained by the vessel in which the cannula was inserted, it is estimated that the collected fluid represents about 10 to 20 per cent of the total.

There are other sources of error such as possible obstruction caused by the cannula, injury at operation, the effect of capillarity, etc. The results given in the table were obtained with the cannulae horizontal, and no allowance was made for capillarity. Cannulae were usually left in until dislodged by movement of the animals.

Taking the total amount of lymph collected (189 cu.mm.) whether obtained by spontaneous flow or massage, and dividing by the total time (18.18 hours), a mean flow of approximately 10 cu.mm. per hour is obtained. Assuming that this is but 10 per cent of the whole, there would be 100 cu.mm. per hour from all the vessels of a whole ear.

These figures may be correlated with those for the area of lymphatics (v. s.), remembering that the possible errors of both still remain. The total mean flow (100 cu.mm. per hour) divided by the total surface area of lymphatics of our assumed average ear (7150 sq.mm.) gives 0.014 cu.mm. of lymph per square millimeter of lymphatic surface per hour.

By similar calculations, the spontaneous flow in the separate experiments varied from 0 to 0.0125 cu.mm. of lymph per square millimeter of lymphatic surface per hour. The greatest continuous massage flow was 0.084 cu.mm. of lymph per square millimeter of lymphatic surface per hour.

DISCUSSION

It is quite likely that the calculations of surface areas presented are understatements of the actual areas. Especially is this probable in computations made on the fixed specimens. In them the values for both surfaces of the ear are but a little higher than for the one surface studied in the living. In the numerical expression of the surface area of the lymphatics, the smaller figure was used, so that the given ratios of rate of flow per unit area are probably high.

For such an extensive endothelial surface, the amount of lymph flow in the lymphatics seems exceedingly small, especially when the size of the blood vessels and the amount of blood vessels and the amount of blood flowing through them are compared. It is, however, in general agreement with studies of lymph flow in peripheral parts of the body, such as the extremities, where investigators have found little or no movement of lymph in the resting animal (compare Starling, '09; White, Field and Drinker, '33; Drinker and Field, '33). Attention is again called to the possibilities of error in the quantitative expression of the lymph flow.

It is interesting that my finding regarding the relative sizes of blood vessels and lymphatics in a given area of the ear agrees closely with somewhat similar measurements made by Drinker and Field ('32) in the web of the frog's foot. They also found the lymphatics and blood vessels approximately equal in area.

The factors which have been observed to cause movement of lymph are listed. It will be noted that all of them are passive, in so far as the lymph vascular system itself is concerned. However, movement of particles was frequently observed which could not be assigned definitely to any of the accessory factors mentioned. The muscular factor in the propulsion of lymph must be negligible in peripheral vessels, because of the extreme scantiness of smooth muscle cells on such vessels. The questions of filtration, osmosis, diffusion, and secretion, and the elasticity of the endothelium as factors concerned with the movement of lymph in lymphatic vessels, have not been directly studied in this investigation.

Since these studies were carried out, the 'preformed tissue' chamber as used in them has been modified by the addition of a splint and shield which greatly reduces trauma to the tissue in the chamber. It seems that, unlike the old, the improved chamber offers little or no obstruction to the lymphatics at the periphery of the 'platform.' In a personal communication, Dr. E. R. Clark informs me that in the improved chamber he has observed erythrocytes within the lymphatics move onto the 'platform' from the periphery and in the reverse direction with no apparent impediment. Since the chief difference in the two consists of the greater amount of new tissue formed in the older type of chamber in reaction to minute injuries, it is suggested that this factor alone may be sufficient, in a confined space, to interfere with the movement of lymph in the lymphatic.

With regard to the openings in lymphatics, the injection experiments reported have proven that, following the insertion of a chamber, which involves injury to lymphatics, definite openings may be present which connect their fluid content

with the fluid outside. These may persist for as long as 13 days. This time may have been prolonged somewhat by the force of the injection or by the presence of the foreign material.

It should be emphasized that all of the openings seen were between the lymphatics and the layer of fluid over the top of the tissue, between the tissue and the mica cover. The significance of these openings has already been discussed in detail (Clark and Clark, '33).

SUMMARY

1. The rabbit's ear possesses a rich lymphatic supply of both superficial and deep vessels, with eight to twelve collecting vessels, which follow the course of the main blood vessels.

2. The vessels of the inner side of the ear pass through the cartilage to join those of the outer side.

3. The total area of the lymphatics of the ear as shown by surface projection is approximately equal to that of the blood vessels.

4. By the introduction of cannulae into lymphatic vessels draining the ear, it was possible to secure data regarding the amount of lymph flow. This was found to be variable, but always extremely small, even when massage was freely used.

Calculations indicated figures ranging from 0 to 0.0125 cu.mm. per square millimeter of lymphatic surface per hour, spontaneous flow, and to a maximum of 0.084 cu.mm. per square millimeter of lymphatic surface per hour after massage.

5. Studies of injected granules indicate that there was some obstruction of lymph flow in the thinner but none in the thicker parts of the older type of 'preformed tissue' chamber.

6. By injected granules it was possible to prove definitely that the suspected openings between lymphatics and the supernatant fluid, seen following the installation of the 'preformed tissue' chambers, are real openings into lymphatics, and that they may persist for as long as 13 days.

These studies were made at the suggestion and under the direction of Dr. Eliot R. Clark, whom I wish to thank for his interest and assistance. I wish to thank Dr. R. G. Williams also, for his many helpful suggestions, especially in matters of technique.

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THE ABSORPTION OF INDIA INK BY THE MESONEPHRIC EPITHELIUM OF NECTURUS MACULOSUS

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ONE TEXT FIGURE AND ONE PLATE (SIX FIGURES)

Two types of renal tubules are present in the mesonephros of Necturus: ventral, primary and secondary tubules, connected with the body cavity by nephrostomes and peritoneal canals; and other tubules, more dorsal in position, which lack coelomic connections (Chase, '23). Thus materials introduced into the peritoneal cavity reach directly the lumina of the tubules of the first type. This direct route was utilized earlier (Dawson, '25, '27) in a study of the reaction of the renal epithelium to solutions of iron salts and to an acid colloidal dye, trypan blue. The present study deals with the reaction to a colloidal suspension of carbon in the form of Higgin's waterproof India ink.

MATERIALS AND METHODS

Mature specimens of Necturus maculosus were used. The India ink was diluted with three volumes of distilled water and filtered twice. One animal was given a single injection of 2 cc. and killed 17 days later. A second received a series of six 0.5 cc. injections over a period of 16 days and was killed after 6 days, while a third was killed 17 days subsequent to the last of a series of eight 0.5 cc. injections distributed over a period of 34 days. The results in each case were very variable, and since there did not appear to be any correlation between either the amount or the duration of the

injections and the distribution of carbon in the mesonephric epithelium, the experiments were not extended.

Blood samples were taken at intervals to determine whether the carbon was being transported from the peritoneal cavity to the vascular system, and portions of the mesonephros and liver were fixed in Helly's solution for histological study. In addition, two animals were injected intravascularly by way of an afferent branchial artery. Each received two successive daily injections of 0.25 cc. of the suspension and were killed 24 hours later. Spleen, mesonephros, liver and heart were fixed, and the blood was studied both in supravital preparations and in smears.

OBSERVATIONS

a. After intraperitoneal injections

Direct absorption of free carbon particles from the peritoneal cavity is accomplished by the mesothelium of the mesenteries and the ventral surface of the mesonephros (fig. 7). Many of these cells round up and are desquamated into the coelom. The macrophages of the peritoneal fluid are numerous and the mesonephros becomes densely infiltrated by these carbon-laden elements. They are especially aggregated in peristomal areas (fig. 2) but do not appear to enter by way of the peritoneal canals. Many are more diffusely distributed in the more dorsal intertubular connective tissue. Others infiltrate and partially disrupt the renal epithelium of the proximal convoluted tubules, frequently occupying the lumina of the tubules and even the capsular spaces (figs. 4 and 6).

Large quantities of free carbon also enter the lumina of the primary and secondary tubules directly by way of the nephrostomes and peritoneal canals, although Lucas and White ('32) report that carmine grains and other solid particles are effectively prevented from entering the nephrostome by the action of the cilia which are arranged at a tangent to the lumen. The carbon does not accumulate in the lumen of the proximal convolute, but is swept on in part by ciliary activity as far as the distal convolute where dense

masses are frequently observed within the tubule. When desquamation of cells occurs in the proximal convolute, the lumina of the distal convolutes are frequently completely plugged with carbon and disintegrating renal cells. Possibly intratubular macrophages may also contribute to the formation of the cellular plug. When this blockage occurs the capsules are greatly distended (fig. 6) both by the fluid eliminated from the glomerulus and that carried in from the coelomic cavity by way of the nephrostomes. The nephrostome cilia exert a greater pressure than the cilia of the neck and accordingly can force coelomic fluid into the capsule (White, '29; Lucas and White, '32).

The epithelium of only two portions of the tubule, proximal convolute and parietal wall of the capsule, was found to react to the free carbon brought into direct contact with the surfaces of the cells by way of the peritoneal canals. The absorption of carbon by the cells of the proximal convolute occurred regularly, while in only two instances were ink particles observed in the wall of the capsule.

The amount and distribution of carbon within the cells of the proximal convoluted segment varies greatly. All portions from the ciliated neck to the intermediate ciliated segment appear to be active in absorption of the particulate material. The more typical patterns of distribution are illustrated diagrammatically in figure 1 and the maximal storage noted is shown in figure 5. In some cases the carbon particles are closely aggregated or densely massed, while in others they may be diffuse and scattered. No particles were ever found in transit through the brush border. After passing this marginal differentiation, the carbon apparently accumulates first in the submarginal vacuoles (fig. 1 a) which are a constant feature of proximal-tubule cells (Curry, '29). Later they may occupy a supra-nuclear position, either forming a diffuse cap (fig. 1 b) or being massed to form irregular bodies (figs. 1 c and 3) simulating in some instances the patterns of Golgi material. Circumnuclear distribution is frequently seen (figs. 1 d, f) and basal accumulations are not

uncommon (figs. 1 e, 3). In some cells almost the entire cytoplasm is filled with carbon (fig. 5). There was no evidence that any of the absorbed carbon was eliminated. The process appeared to be one of progressive storage.

Intercellular carbon is frequently present. It is localized in the processes of macrophages which migrate actively through the proximal-tubule walls. Sloughing of epithelial

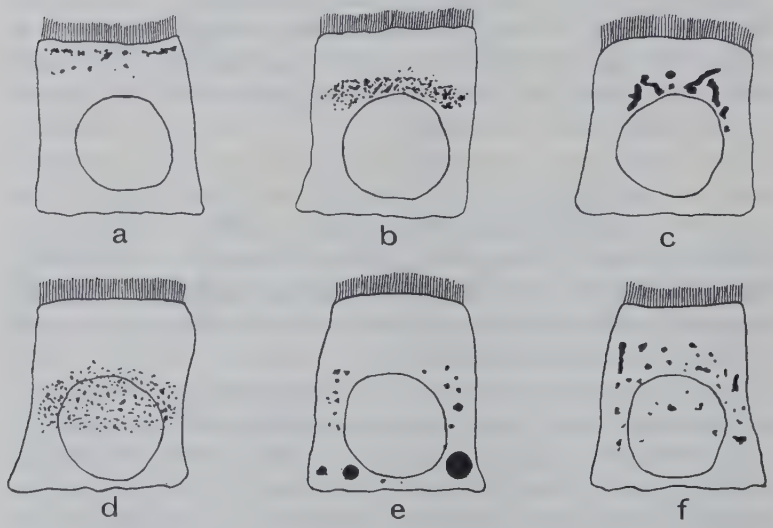

Fig. 1 A series of sketches to illustrate the varying amounts and distribution of India ink within the cells of the proximal convoluted segments of various primary mesonephric tubules.

cells is also common and usually involves cells which have absorbed large quantities of ink particles. The epithelium may also be disrupted by the excessive infiltration of macrophages. In tubules in which sloughing has occurred, the remaining cells become flattened and elongated, or even squamous, and mitotic figures are common. The repair of a disrupted wall is illustrated in figure 4. Here it is probable that the infiltration of macrophages was a contributory factor in the loss of the epithelial cells.

The storage of carbon in the outer wall of the capsule of Bowman is puzzling since normally this region would never be exposed to free ink particles. It seems probable that in the two instances noted, the tubules must have been temporarily blocked distally, resulting in the backing-up of ink-containing fluid into the capsule. There was no evidence of storage in the cells covering the capillary tufts of the glomerulus. Ink was never noted in the striated cells of the distal convoluted segment, and the ciliated cells of the nephrostomal plate, peritoneal canal, tubule neck, and intermediate segment were likewise free of ink at all times.

In striking contrast to the observations of Mackmull and Michels ('32) ink remained localized for the most part in the peritoneal cavity and the serous membranes limiting it. Even the liver showed very little invasion. Free carbon never reached any tissues or organs except as described for the mesonephros. Carbon-laden macrophages were rarely seen in the circulation.

b. After intravascular injections

A study was also made of the blood, mesonephros, liver, heart, and spleen of the two animals killed 24 hours after receiving intravascularly, in two successive injections, a total of 0.5 cc. of the carbon suspension. In these animals practically all free carbon had been removed from the circulation and stored, either in the cells of the blood stream or in the phagocytic tissues of the liver and spleen. The endothelium of the glomerular capillaries and of the capillaries surrounding the mesonephric tubules was free of carbon although macrophages were present in the intertubular connective tissue. Since the mesonephric endothelium remained free of carbon while the reticulo-endothelium of both liver and spleen contained masses of ink, these experiments were discontinued. It seemed very improbable that India ink introduced into the circulation could be absorbed by the renal epithelium.

DISCUSSION

The literature on the absorption of colloidal carbon from body spaces, such as the peritoneal cavity, has been adequately reviewed by Mackmull and Michels ('32) and need not be repeated. In Necturus, the carbon leaves the peritoneal cavity much more slowly than in the cunner. Free carbon was found only in the lumina of the renal tubules, and very few macrophages entered the circulation. The invasion of carbon macrophages was greatest in the mesonephros. The liver was only slightly affected.

The chief feature of interest in this study is the absorptive capacity exhibited by the brush-border cells of the proximal convoluted portion of the mesonephric tubule. Permeability to particulate matter in vertebrates is usually associated only with free phagocytic cells and the reticulo-endothelial system, although liver epithelium has been found both to absorb and eliminate carbon (Weatherford, '32). Other observations cited by Weatherford indicate that tracheal, bronchial, and vaginal epithelium of certain mammals may also take up particulate material. The absorption of carbon by the outer wall of Bowman's capsule, when effectively exposed to the suspension of ink following blockage of the tubule with distention of the capsule by peritoneal fluid, is a further expression of the marked permeability of the renal epithelium. Possibly only the epithelium of the first type of tubule can react with particulate matter in this fashion. The limitations of the procedure employed did not permit an investigation of the more dorsal tubules which lack peritoneal connections.

These observations do not contribute directly to the solution of the problem of secretion and reabsorption in the renal tubule, except in so far as they give some measure of the degree of permeability of the brush-border cells under normal environmental conditions.

SUMMARY

Colloidal carbon in the form of diluted Higgin's waterproof India ink, when injected intraperitoneally, reaches by way of the nephrostomes and peritoneal canals the lumina of the primary and secondary tubules of the mesonephros of Necturus. Usually it is absorbed and stored only by the brush-border cells of the proximal convoluted segment. When blockage of the distal segment occurs, the suspension is forced by the ciliary pressure of the peritoneal canal into the capsule; carbon is then stored in the epithelium of the outer wall of the capsule.

The amount of carbon stored by brush-border cells is very variable and could not be correlated with either the amount or duration of the injections. Heavily laden epithelial cells may be desquamated, and the epithelium is repaired by thinning of the cells followed by multiplication by mitosis.

There is an extensive infiltration by carbon macrophages from the peritoneal cavity. These cells accumulate chiefly in the interstitial tissues, but may infiltrate the renal epithelium and in many instances reach the lumina of the tubules and the capsular spaces.

Ink injected intravascularly was found not to reach the mesonephric epithelium. Even the endothelium of the peritubular capillaries had not taken up any carbon after a 48-hour interval although the reticulo-endothelial cells of both spleen and liver contained large amounts.

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PLATE 1

EXPLANATION OF FIGURES

All figures are photomicrographs. The magnification was varied in order to include representative areas and to bring out adequate detail.

2 An area from the ventral surface of the mesonephros, including a nephrostome and peritoneal canal, showing the dense infiltration of the intertubular tissue by carbon-laden macrophages from the peritoneal cavity. Seventeen days after the last of a series of eight injections (0.5 cc.) given over a period of 34 days.

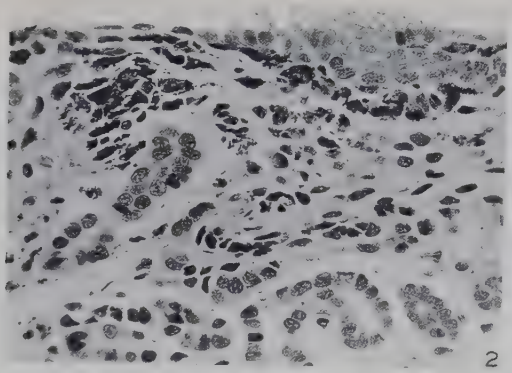
3 Proximal convoluted tubules showing the intraepithelial accumulations of India ink. In the transverse section much of the ink is located basally while in the longitudinal section the ink is massed in a supra-nuclear position. Seventeen days after a single injection of 2 cc.

4 Longitudinal section of a proximal convoluted tubule showing dilatation and repair following desquamation of the epithelium. The cells in the lumen are either macrophages or sloughed epithelial cells filled with ink particles. Six days after a series of six injections (0.5 cc.) given over a period of 16 days.

5 An oblique section of a proximal convoluted tubule showing a maximal intraepithelial accumulation of ink particles. From same animal as figure 2.

6 Distention of the capsule of a primary tubule due to an obstruction of the distal convoluted portion. Several macrophages are in the lumen. From same animal as figure 5.

7 Portion of the renal corpuscle of a primary tubule showing the storage of ink in the epithelium of the parietal wall of the capsule. Peritoneal epithelium and adjoining parietal wall on the left; portions of glomerular tufts with overlying epithelium on the right. From the same animal as figure 6.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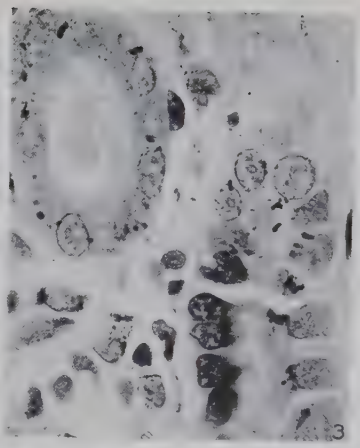
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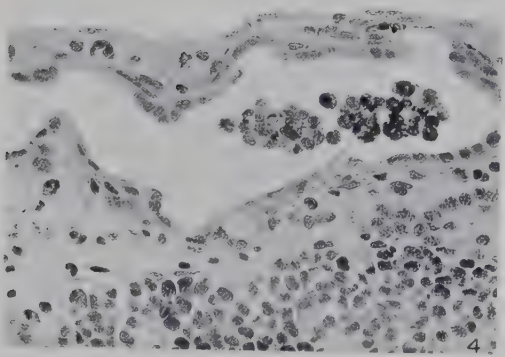
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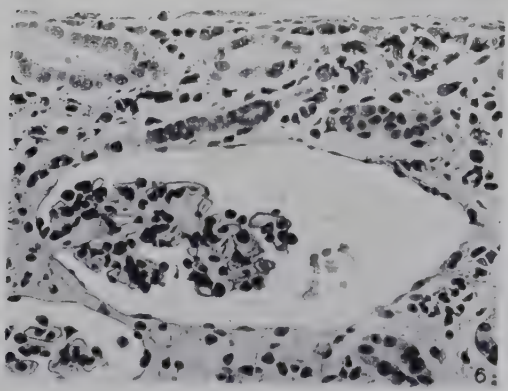
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CYTOLOGICAL CHANGES AFTER AUTONOMIC AND ADRENALIN STIMULATION OF THE CAT'S SUBMAXILLARY GLAND

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TWO PLATES (EIGHT FIGURES)

The salivary glands are under the control of nerve fibers reaching the glands by way of both the sympathetic and the parasympathetic systems. The nature of this double innervation has been a subject of investigation since R. Heidenhain (1878) developed his concept of 'secretory' and 'trophic' nerves to the glands, the former being concerned with the actual extrusion of the secretion and the latter qualitatively influencing the process of formation of the secretion within the cell. This theory and others subsequently advanced (Langley, 1888; Babkin, '13) have not satisfactorily explained all the phenomena. They have proved inadequate largely because the glands were looked upon as single units under a double nerve control whose duplication extended to every cell. Little attention was paid to the possibility that the various distinct epithelial components of the salivary glands might constitute the true functional units acting under their own peculiar controls (sympathetic, parasympathetic, hormonal, etc.). Physiological data have accumulated favoring this hypothesis of intra-glandular units controlled by various mechanisms.

The possible application of this idea to the salivary glands was discussed by Babkin ('31) and the desirability of more histological data was apparent. The observations reported

[1] Part of the work of this investigation was carried on in the Department of Physiology of this University.

here are some of the results of the histo-physiological investigation referred to in that paper. A summary appears in the McGill University Abstracts ('32). In this present report attention is directed to observations of the changes occurring in the demilune and alveolar cells of the submaxillary gland. The reactions of the other secretory elements are at present under study.

Among those who have experimentally activated the submaxillary for histological study two require mention here. Tagaki ('25) investigating the role of mitochondria in gland secretion studied the cytological changes in the submaxillary of the cat, finding among other things that faradic stimulation of the chorda tympani nerve caused changes in the alveolar cells. Stormont ('28) stimulated the sympathetic and parasympathetic nerve supplies to the rabbit's submaxillary gland, showing that the former activated 'tropochrome' and the latter 'homeochrome' cells.

METHODS

The mode of experimentation that we have followed consists in the activation of the submaxillary gland by electrical stimulation of the chorda tympani nerve, and of the cervical sympathetic trunk, and by intravenous injections of a solution of 1/10,000 adrenalin. The animal chosen was the cat because the stimulation of the sympathetic nerve supply to its submaxillary gland, and also adrenalin injection, causes only a short period of vasoconstriction which is followed by vasodilatation (Carlson, '07; Barcroft, '14). This means that the vasomotor effect was practically similar in all the experiments.

The animals were starved for 24 hours previous to the experiment. Anesthesia was induced by intraperitoneal injections of Dial, 0.5 to 0.7 cc. per kilo, and in this way no salivation occurred during induction in most of the cases. In every experiment all nerve supply to both glands was cut, one gland being used as a control and removed at the end of the experiment simultaneously with the activated gland. When adrena-

lin was injected the control was taken at the beginning of the experiment. Sympathetic nerve stimulation was carried out with the electrodes on the cervical sympathetic trunk. In all cases the duct of the operated gland was cannulated and the secretion measured.

The fixatives chiefly relied upon were formol-Zenker and Kolster's. The usual stain was a combination of iron haematoxylin and mucicarmine.

RESULTS

a. Stimulation of the chorda tympani

Six experiments were performed, the time of stimulation varying from ½ to 3 hours and resulting in a free and copious flow of saliva that ranged from 2 to 47 cc. Sections of the tissue show the alveolar cells decreased in size and having an irregular cell architecture. These changes increase in degree with the total duration of effective stimulation. In some areas the lumen of the alveolus is increased in size so that the demilunes appear to border directly on it. These observations agree in general with those of other workers, e.g., Tagaki. In one case the stimulation at 10-minute intervals was prolonged for 3 hours resulting in 47 cc. of saliva. The alveolar cells of its stimulated gland show marked irregularity in size and shape (figs. 1 and 2) and their contents show a great variability in the degree to which they take up mucicarmine, many areas showing practically none. The nuclei of the cells are enlarged, becoming more spherical and less dense so that they appear similar to the nuclei of the demilune cells.

In the demilunes no definite or constant change can be recognized. As the effects on the alveolar cells becomes more marked the demilunes enlarge, the nucleus becomes less dense and the cytoplasm more finely granular. This change does not appear until the alveolar cells have suffered very intense stimulation and may be due to the purely mechanical cause of diminished side pressure. In the case already mentioned in which stimulation resulted in 47 cc. of saliva, this change in the demilunes is quite apparent (figs. 1 and 2) and when

combined with the nuclear and cytoplasmic changes noted as occurring in the alveolar cells, in some areas it is difficult but not impossible to differentiate them.

In all the experiments in this group, the demilune cells never exhibit more than rare and isolated cytoplasmic vacuolation in sections from either stimulated or normal tissue.

b. Stimulation of the cervical sympathetic trunk

Eight animals were subjected to stimulation of the cervical sympathetic trunk. It requires careful grading of the intensity of the stimulus and cautious manipulation of the nerve to secure a continued secretory response with this type of stimulation. It is also important that there be some means of determining the precise output of the gland under this type of excitation since our experiments have shown that the gland may fail to give a secretory response while the stimulation is still capable of effecting the pupil by way of the pupillodilator fibers in the sympathetic trunk. The rate of the scanty secretion is best studied by means of its effect on the progress of a bubble in a graduated tube attached to the cannula. Using such means it was possible to register successful stimulation of the gland for periods of from 2 to 3 hours, resulting in from 2 to 5 cc. of saliva.

In the sections no definite changes can be seen in the alveolar cells. The demilunes, however, show distinct clear vacuoles in their cytoplasm (figs. 3 and 4). These vacuoles are scarce and not marked where the stimulation has been of less duration than 2 hours and has resulted in less than 3 cc. of saliva. They are always more marked in the stimulated gland than in the control since the controls in all cases show vacuolated cells rarely or not at all. A peculiar feature of this change in the demilunes is that it is not uniform in its occurrence, but is found in scattered areas throughout the gland. A careful comparison between the activated gland and its control shows, however, that the demilunes have suffered a quite evenly spread, moderate but definite decrease in size. Less general and less marked is an increase in the size of the nucleus and a decrease in its density.

Since vacuolation might represent a preliminary stage in the extrusion of the secretion, it was thought that any obstacle to the elimination of the secretion would increase the formation of these vacuoles. With this in view one experiment was arranged so that the stimulated gland had to work against the pressure of a clamped tube. Stimulation was continued for an hour. The slides show a very marked vacuolation of the demilune cells (figs. 5 and 6). It is evenly spread and the vacuoles vary in size from medium to large.

c. Stimulation by adrenalin

On account of the sympathicomimetic action that it exerts, three animals were injected intravenously with adrenalin in doses starting at 1 cc. of a 1/10,000 solution and being increased as the secretory response slackened. The drug was administered at 5-minute intervals over periods of from 1½ to 2 hours. The total injections in the three experiments were 21, 23, and 36 cc. They were made into the femoral vein and almost immediately caused a quickened heart beat and about 15 to 20 seconds later a secretion from the gland. The rate and amount of flow was very similar to that obtained by sympathetic stimulation. In the first case the total output was 3 cc., in the second 5 cc., while in the third it was not measured accurately but was estimated to be in excess of the other two.

The result upon section is apparently identical with that following sympathetic stimulation. The alveolar cells undergo practically no change, but the demilunes present definite cytoplasmic vacuoles of varying degree and number. They are more evenly distributed than in the case of sympathetic stimulation, but on the other hand, changes in the size of the cells or in their nuclei are not so definite. The control glands of the first two animals are similar in appearance to those from all other experiments. In the third case the control gland is unique among all since the demilunes exhibit a scattered vacuolation of a moderate degree. Throughout the whole of the gland they are of much smaller size than is

usually found (fig. 7). They appear very much as though they had been subjected to sympathetic stimulation. No explanation of this finding can be offered, but it gives rise to interesting speculations as to what constitutes a 'normal' gland and what extent various ways of handling an animal immediately previous to anesthesia may vary that normal state. However, in this case the stimulated gland shows a striking increase in the amount and degree of its vacuolation when compared to the control (figs. 7 and 8).

DISCUSSION

This investigation establishes the fact that different and peculiar cytological changes in the cat's submaxillary gland follow upon stimulation of the parasympathetic and sympathetic nerve supplies to the gland.

In the case of the action of the parasympathetic, the effect is exerted predominantly on the alveolar cells. This action had been noted by Langley (1898), Tagaki ('25), and others, but there is no definite statement that the demilunes undergo no significant change. It does not appear, however, that precautions have been taken to exclude by section the action of the sympathetic, an omission that might confuse the histological picture.

A different finding results from sympathetic and adrenalin stimulation. The alveolar cells appear to be practically exempt from change. The demilunes, on the other hand, show vacuolation of their cytoplasm and, to a lesser extent, changes in the size and density of the nucleus. The significance of vacuolation of the cytoplasm in the secretory cycle of a gland cell cannot be definitely stated. Whether or not it represents a preliminary stage in the actual extrusion of the cell secretion in response to intense stimulation, nevertheless it may be conceded that any significant difference in the number and size of vacuoles in certain cells of a stimulated gland when compared with a control gland from the same animal, can be taken as evidence that the particular type of stimulation is exerting some effect, either direct or indirect, on such cells.

Histo-physiological investigation of the sympathetic nerve control by means of adrenalin and electrical stimulation seems to have been somewhat neglected as a method of study in relation to the submaxillary gland of mammals. Stormont used electrical stimulation with the rabbit's submaxillary gland describing the sympathetic action on 'tropochrome' cells, but no record of the utilization of a similar procedure using the cat's submaxillary nor the use of adrenalin in regard to its action on salivary secretion has been found.

This investigation also supports the modern view that the demilune cells are an independent cell type rather than functional or developmental stages of alveolar cells as in the older theories (Zimmerman, '27). In view of the similarity of appearance between demilune and alveolar cells after long continued stimulation of the chorda tympani it is easy to understand the genesis of the 'Phasentheorie.'

While from our results we would ascribe to the parasympathetic and sympathetic different effects exerted predominantly on different cell types, we would not say that their effects are rigidly confined to such types. The different secretory cells are in close connection and it is not difficult to suppose that any one cell in activity would influence its neighbors by virtue of that activity. Such an influence is more easily understood in the light of the modern theory of nerve atcion through the formation of local hormones.

SUMMARY

Electrical stimulation of the chorda tympani in the cat causes changes in the alveolar cells of the submaxillary gland, while similar stimulation of the cervical sympathetic trunk causes the appearance of cytoplasmic vacuolations in the demilune cells. This effect of the sympathetic is duplicated by administration of adrenalin. The findings support the hypothesis that the parasympathetic and sympathetic nerve supplies to the gland each primarily affect different gland elements.

I desire to record my appreciation of the help given by Profs. B. P. Babkin and J. C. Simpson.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

The figures are all photomicrographs × 400, taken from sections stained with iron haematoxylin and mucicarmine.

1 Area of control gland, animal 3.

2 Area of stimulated gland, animal 3. Chorda tympani stimulation for 3 hours; 47 cc. of saliva. Though the demilune cells are a little larger and have less dense nuclei than in the control, they show no vacuoles. The mucous cells are irregular and show a variable degree of mucicarmine absorption.

3 Area of control gland, animal 5.

4 Area of stimulated gland, animal 5. Sympathetic stimulation for 2 hours; 5 cc. of saliva. The demilunes show vacuoles.

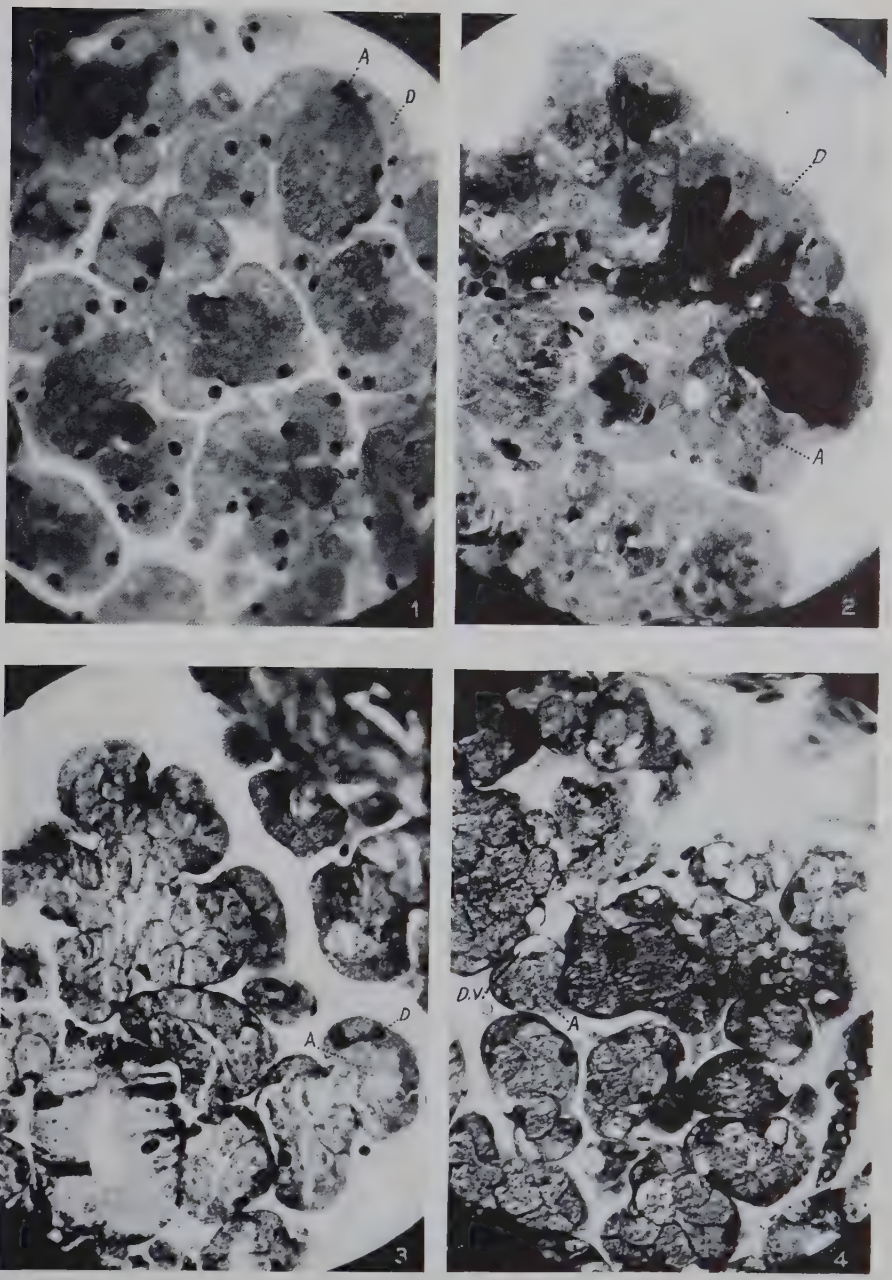

A
D
1
D
A
2
A
D
3
D.V.
A
4

PLATE 2

EXPLANATION OF FIGURES

5 Area of control gland, animal 16.

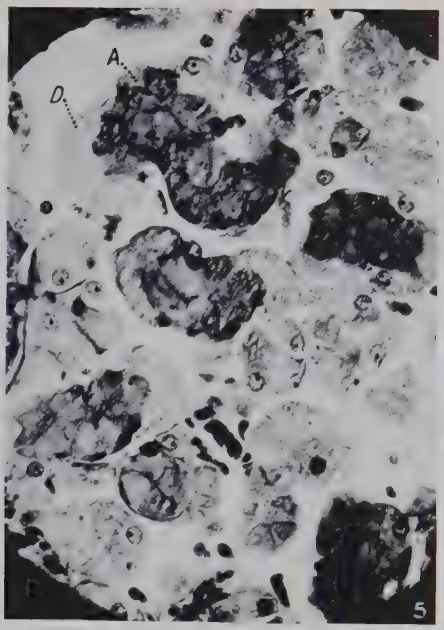
6 Area of stimulated gland, animal 16. Sympathetic stimulation for 1 hour; secretion against pressure. Many of the demilunes are vacuolated.

7 Area of control gland, animal 11. Demilunes smaller than usual.

8 Area of stimulated gland, animal 11. Adrenalin injections over a period of $1\frac{1}{2}$ hours. The vacuolation of the demilunes is widespread and quite marked in degree.

ABBREVIATIONS

A., alveolar cell
D., demilune cell
D.V., vacuolated demilune cell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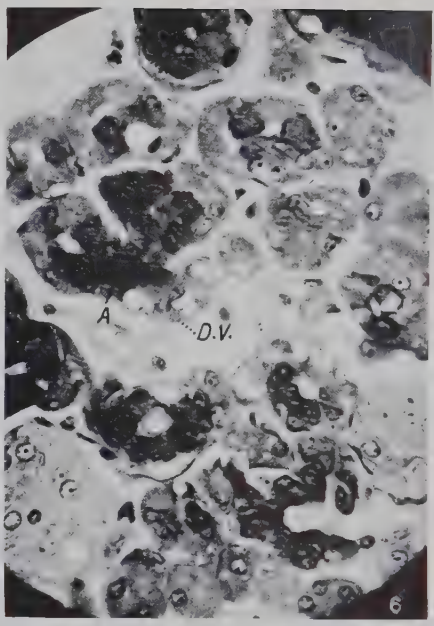
A
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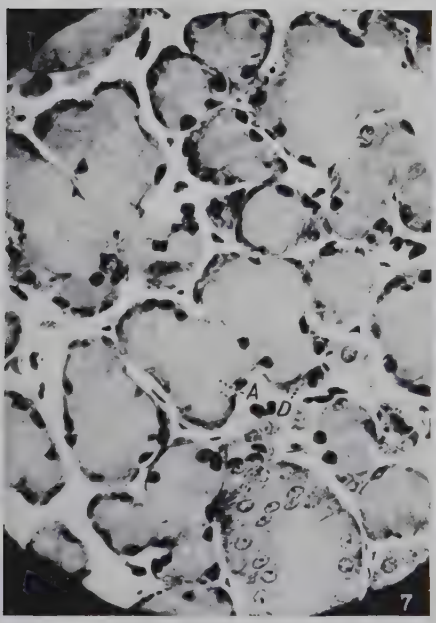
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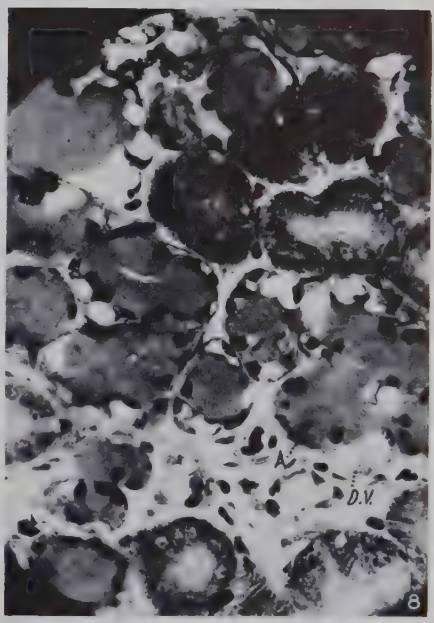
A
D.V.
8

AN EXPERIMENTAL STUDY OF THE RELATIONSHIP OF FLASK CELLS TO MOLTING IN TRITURUS VIRIDESCENS

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FIVE FIGURES

INTRODUCTION

Flask cells were first reported by Rudneff (1865) in the skin of the frog. F. E. Schulze (1867) described them in the skin of some of the urodeles, and Pfitzner (1880) in Salamandra maculosa. These cells have since been found in the skin of nearly all Amphibia.

Various functions in connection with molting have been ascribed to the flask cells. Pfitzner (1880) thought that in Salamandra maculosa they had a mechanical function of holding on the cornified layer of the epidermis and were pulled out with this layer at the time of molting. P. Schultz (1889) concluded that the flask cells were glandular, and served to loosen the superficial layer at the time of molting by secreting a substance under it. Each cell receded as it produced its secretion, leaving a hyaline plug in the differentiating horny layer. Dennert ('24) states that in Salamandra maculosa and several other urodeles the flask cells are not pulled out when the cornified layer is shed, but withdraw from this layer just before molting. After the molt the adjoining cornifying cells grow up over the exposed end of each flask cell. Dennert suggests that the flask cells may hold on the cornified layer, and their withdrawal, perhaps supplemented by secretory activity, may release it.

Fahrenholz ('27), working on the skin of Xenopus calcaratus, decided that if the flask cells played any role in molting, it was that of holding the cornified layer to the underlying layers of the skin until the onset of molting. He describes a periodic cornification of the outer parts of the flask cells simultaneously with the cornification of successive layers of epithelium. At every molt the cornified portion of each flask cell is cut off and a new head and neck formed. Thus the flask cells are not covered by the epidermal cells, but lie beneath cornified portions of their own cytoplasm. These isolated portions of the flask cells, Fahrenholz states, are recognizable in mounts of molted skin as round, light areas between the boundaries of the flattened epithelial cells.

The recent work of Adams, Kuder, and Richards ('32) on induction of molting in Triturus viridescens by administering anterior-pituitary to stimulate the thyroid and on inhibition of molting by thyroidectomy, together with the work of Adolph and Collins ('25) on artificial induction of molting in the same animal by mechanical stimuli, suggested a means for the study of the relationship of flask cells to molting. If, as both Fahrenholz and Dennert suggest, the flask cells serve to hold the horny layer until molting occurs, it might be expected that in thyroidectomized animals, in which there is an accumulation of cornified epithelium, the flask cells would show changes in form or number. On the other hand, if they bring on molting by secreting a substance under the horny layer, as suggested by Schultz, there should be some indication of their increased function in animals in which molting is stimulated by mechanical means or by administration of anterior-pituitary.

MATERIALS AND METHODS

The animals used in this study were adult Triturus viridescens. A general survey of the skin from the neck, fore and hind limbs, back, abdomen, and dorsal tail fin showed little regional difference in the number of flask cells. Therefore, skin from the dorsal tail fin was used in all studies, since small pieces could be readily removed from this region, and

the skin of the same animal examined under various conditions.

The following methods were used: Successive injections of minced anterior-pituitary of frog into the abdominal cavity until the animals showed degnite signs of activation; removal of both thyroid glands; mechanical stimulation of molting in normal and pituitary-activated animals; and stimulation of thyroidectomized animals by the addition of a small amount of desiccated thyroid to the water.

For all operations, including removal of successive pieces of tail fin, the animals were anesthetized in a 0.1 per cent aqueous solution of chloretone. All tissues were fixed in Helly's fluid, followed by a 48-hour post-chromation at 35°C. Sections 4 μ thick were cut in paraffin and stained in Regaud's iron haematoxylin. Whole mounts of molted skin were made in some instances. The skin was fixed in 10 per cent formalin and stained in Delafield's haematoxylin and eosin.

In comparing the numbers of flask cells in different pieces of skin, counts were made of the cells in seven 4 μ sections. In each section, 1.6 mm. of skin, from the dorsal tip of the fin downward on one side, was examined, so that in each piece of tissue the flask cells were counted in an area 1.6 mm. long and 28 μ wide.

ANTERIOR-PITUITARY ACTIVATION

Eight animals were used for anterior-pituitary activation. Toward the end of the treatment, the females were so much activated that they would lay eggs after each injection, and the males showed greatly enlarged tail fins, swollen cheek glands, and black pads on the toes and femoral surfaces of the hind limbs (Dawson and Jimenez, '33). The skin was smooth and glossy, and molting often occurred within a short time after injection of the anterior-pituitary. At the end of the injections, a piece of the dorsal tail fin was removed from each animal.

Molting was further stimulated in four of the activated animals by fastening a silk thread around the abdomen with

one stitch in the back. As described by Adolph and Collins ('25), molting occurred from 48 to 96 hours after such stimulation, and the animals continued to molt as long as the thread remained. After a series of three to five molts over a period of 11 days, a second piece of tail fin was removed from each animal. The molted skin was kept for study.

THRYOIDECTOMY

Both thyroid glands were removed from six salamanders. The method used was that described by Adams, Kuder, and Richards ('32). At the end of 2 weeks the animals showed definite effects of thyroidectomy.

Portions of tail fin were removed from all animals at the end of 33 days, again from four at the end of 49 days, and from the other two after 61 days. Following the removal of the second samples, treatment with desiccated thyroid was begun. Six days later, when all the animals had either molted or showed loosening of the skin, pieces of tail fin were again taken.

FORCED MOLTING OF NORMAL ANIMALS

Pieces of tail fin were removed from three untreated animals which had been kept in the aquaria for a period of several weeks. These animals were then stimulated to molt by use of a thread around the abdomen. After the induction of three to five molts over a period of 12 days, more tissue was taken.

DESCRIPTION

Flask cells in the skin of the controls

The flask cells of the skin of Triturus correspond closely to Fahrenholz's general description of flask cells of Amphibia. The epidermis of the control animals consists of four or five layers of epithelial cells. The rounded part of the flask cell, containing the nucleus, is usually found at the level of the third cell-layer from the dermis, occasionally in the second, more rarely in the fourth. An elongate neck, some-

what narrower than the body of the cell, extends toward, but rarely opens upon the free surface of the epithelium. Ordinarily it is covered by the cornified layer, and frequently by the underlying layer, 'Ersatzschicht' of Fahrenholz, which replaced the cornified layer at the time of molting. The cytoplasm in the neck of the flask cell is often finely striated or granular.

Changes in the flask cells resulting from experimental procedure

In the animals in which molting had been stimulated by injections of anterior-pituitary, the epidermis was reduced in thickness to three or four layers of cells. The flask cells differed only slightly from those described as normal, the neck being somewhat shorter and broader. The level of the cell bodies remained unchanged. The necks were found to end either below the cornified layer or beneath the underlying layer, as in the normal condition (fig. 1).

Adams, Kuder, and Richards have recently shown ('32) that the number of uncornified layers, as well as the number of cornified layers, was increased after thyroidectomy. Similar results were obtained in the present investigation. In the sections of tail fin from thyroidectomized animals, the uncornified portion was sometimes seven cells thick, instead of the usual four or five, and as many as eight cornified layers had accumulated. In such thickened epithelia the bodies of the flask cells remained at their normal level, while their necks were greatly elongated, extending as in all other cases to the cornified or underlying layer (figs. 2, 3). In these long, slender cells, the outer end of the neck often showed the same staining reaction as that of the cornified layer. This reaction was encountered occasionally in the flask cells of the control animals and of those stimulated with anterior-pituitary, but was seen more frequently in thyroidectomized animals. It may have been this cornified appearance of the neck that led Pfitzner to think that the flask cells were an integral part of the cornified layer of the skin, and that they were pulled out at molting.

Following the period of forced molting by mechanical stimulation, the flask cells were shortened and broadened in both the control and pituitary-stimulated animals. The shortening was obviously the accompaniment of thinning of the epidermis, which in the pituitary-stimulated animals was reduced to a thickness of two or three cells. In the latter animals, the flask cells were often so much shortened as to have scarcely any neck portion, since the nuclei retained their usual position in the second or third layer from the dermis.

In thyroidectomized animals, molting induced by addition of desiccated thyroid gland to the water restored the flask cells to a condition resembling that of the controls. The necks

Figures 1, 2, and 3 are camera lucida drawings of flask cells from sections of tail fins, × 890. Figures 4 and 5 are camera lucida drawings of whole mounts of molted skin, × 290.

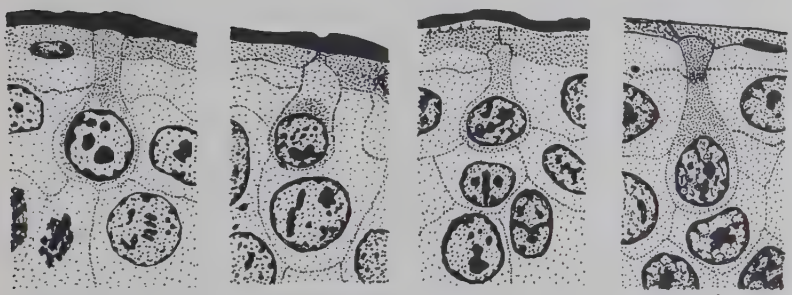
Fig. 1 Series from the skin of anterior-pituitary stimulated animals, showing the separation of the cornified layer from the flask cells by the pushing in of the cells under the cornified layer. a, Flask cell extending to the under surface of the cornified epithelium. b, The cells under the cornified layer are pushing in, separating this layer from the end of the flask cell. c, The cornified layer is completely separated from the end of the flask cell. The boundary between the two cells which have brought about this separation can be distinctly seen between the end of the flask cell and the cornified layer. d, The superficial layer is not completely cornified. The boundary between the overlying cells can be seen above the flask cell, showing that this cell has been covered by the overgrowth of the cells which are to become cornified.

Fig. 2 Flask cell and surrounding cells from the skin of an animal thyroidectomized for 33 days. The layers of cornified epithelium have been retained. Note the series of depressions in these layers above the flask cell, showing where the end of the cell rested in each layer. Note also the elongation of the neck of the flask cell.

Fig. 3 Skin from the same animal as that shown in figure 2, 49 days after thyroidectomy. Note the greater elongation and narrowing of the neck of the flask cell, and the darkly stained outer portion.

Fig. 4 Molted cornified epithelium from an animal stimulated with anterior-pituitary. The points of contact with the flask cells show as dotted circles. The cell boundaries cross these areas.

Fig. 5 Molted cornified epithelium from an animal stimulated with anterior-pituitary. The epithelium was shed before the flask cells were completely covered. The spaces previously occupied by the tips of the flask cells appear as openings in the molt. The stippled areas represent slightly elevated areas around the openings, and indicate the amount of overgrowth at the time of molting.



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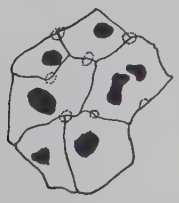

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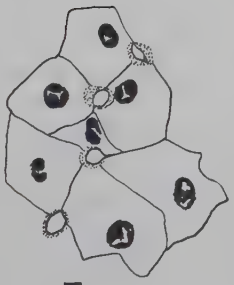

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became shorter and broader, and the number of cell-layers in the epidermis was reduced.

Relation of the flask cells to the cornified layer of epidermis

With the exception of occasional cases found after rapidly successive molts, the tips of the flask cells are covered by the cornified layer of the epidermis, and often also by the underlying layer. The manner in which the ends become covered appears to differ from that described by Dennert for Salamandra and Triton, and by Fahrenholz for Xenopus. In Triturus the ends of the flask cells become covered either before the molt by the growth over them of the cells of the layer below the cornified layer, or after the cornified layer has been cast off. Regardless of the time, the ends are eventually covered by the growth over them of the cells of either side. In mounts of the cast skin, it may be seen that the boundaries of the adjacent cells have come together above the flask cells. The former locations of the ends of the flask cells are marked by circular, light areas representing slight depressions in the under surface of the cornified epithelium (fig. 4). In one animal, in which several molts occurred in rapid succession, the cornified layer was shed before the process of overgrowth had been completed, leaving holes in the cast skin through which the ends of the flask cells had protruded. That partial overgrowth had occurred is indicated by raised areas surrounding the holes (fig. 5). Supplementary evidence that the flask cells are covered by the growth over them of the surrounding cells was obtained from sections. A series of stages illustrating this process is shown in figure 1.

Occasionally in the skin of a thyroidectomized animal in which several layers of cornified epithelium have been retained, each layer shows the depression on its under surface where the tip of the flask cell originally tested. Each layer has been successively separated from the flask cells by the pushing in of the underlying cells (fig. 2).

In some cases, the flask cell has the appearance of having withdrawn from the cornified layer preceding overgrowth by the underlying cells, as described by Dennert in Salamandra (fig. 3). This withdrawal may result from the shrinking of the flask cell or from accumulation of secretion from the flask cell, or it may be due to the pushing up of the cells on either side. Since in the majority of instances the flask cells seem to become separated from the cornified layer by the pushing in of the adjacent underlying cells, the latter suggestion would seem the more probable. There seems to be no definite set of conditions that determines the appearance of the spaces above the flask cells, for they were observed alike in actively molting animals and in those in which molting had been inhibited.

There is no evidence of the separation of the outer end of the flask cell to form a plug between adjacent cornified cells, as described by Fahrenholz in Xenopus. In the shed skin, with the exception previously noted, the boundary between the cornified cells can be seen to extend through the center of the depression caused by the end of the flask cell, rather than to pass around a plug formed by the retention of the severed end of the flask cell in the molted layer. In some instances a dark line crosses the neck of the flask cell, resembling figures of Fahrenholz which illustrate the formation of a new head beneath the old one (fig. 1 d). In the figure, however, it will be noted that the same flask cell has been covered distally by the overgrowth of two adjoining cells, the boundaries of which show distinctly.

Number of flask cells

The number of flask cells in a given area of skin chosen at random within the sectioned part of the excised piece of tail fin varied greatly in different individuals. However, the number found in any one individual after forced molting corresponds closely to that found before forced molting. In the accompanying table the number of flask cells from the given area in each of the animals studied is recorded. The

wide variation among the individuals in each set renders the average numbers from so small a group insignificant. The numbers for animal *6* of the pituitary-stimulated animals were not included in calculating the averages because the sections made from the first material were cut so obliquely as to make the count inaccurate.

TABLE 1

Number of flask cells in area 1.6 mm. by 28 μ

PITUITARY-STIMULATED ANIMALS	AT END OF INJECTING PERIOD	AFTER MOLTING INDUCED
1	17	
2	6	7
3	30	
4	45	44
5	13	16
6	(20)	(47)
7	21	23
8	12	
Average	21	22
THYROIDECTOMIZED ANIMALS	**MOLTING INHIBITED**	**MOLTING INDUCED BY ADDING THYROID**
1	25 (61 days)	18
2	38 (61 days)	47
4	40 (49 days)	33
5	62 (49 days)	58
7	14 (49 days)	14
8	46 (49 days)	45
Average	37	36
CONTROL ANIMALS	**BEFORE MOLTING**	**AFTER MOLTING INDUCED**
1	52	57
2	22	26
3	44	36
Average	39	39

SUMMARY

The possible relationship of flask cells of the epidermis to molting in Triturus viridescens was investigated experimentally. In animals activated by anterior-pituitary, thinning of the epidermis was accompanied by shortening and broadening of the necks of the flask cells. Following thyroid-

ectomy, the great increase in thickness of the skin, involving both cornified and uncornified layers, was accompanied by elongation and narrowing of the necks of the flask cells. Forced molting, in reducing the number of cell-layers in the epidermis, brought about a shortening and broadening of the necks of the flask cells in normal, thyroidectomized, and pituitary-stimulated animals.

The flask cells were very stable under the experimental conditions to which they were subjected. The bodies of the cells tended to retain their position in the second or third cell layer from the base of the epidermis, regardless of the total number of cell layers. The necks of the cells always extended to the under surface of the cornified layer or of the underlying layer.

The ends of the flask cells are covered, usually before molting occurs, not by the isolated heads of the flask cells themselves, as described by Fahrenholz in Xenopus, but by the overgrowth of the cells of the layer under the cornified layer, as Dennert found in Salamandra.

Although a small depression or vacuole was often seen between the end of a flask cell and the cornified epithelium, this space was not shown to have been produced by a secretion of the flask cell. It seems probable that such spaces might be caused by the pushing up of the surrounding cells, anticipatory to the covering of the ends of the flask cells.

No change in the number of flask cells occurred as the result of activation with anterior-pituitary, of thyroidectomy, or of forced molting by mechanical stimuli.

That the separation of the cornified layer of epithelium from the flask cells is not the causative factor in the molting of this layer is demonstrated by the fact that in thyroidectomized animals many cornified layers may be retained, when all but the lower one have lost their contact with the flask cells.

The flask cells apparently have no direct influence on molting but their tips always maintain an intimate contact with the layer undergoing cornification. Their function remains obscure.

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NEW BOOKS AND PUBLICATIONS

NOTICES BY F. E. WELLS

HISTOLOGY, by S. Ramón-Cajal. Revised by J. F. Tello-Múñoz. Translated from the tenth Spanish edition by M. Fernán-Núñez. 1933. Size, 6¾ × 9¼ inches. Pages xiv + 738. 535 illustrations. Index. Cloth. Price, $8.00. The Williams & Wilkins Company, Baltimore.

All English-speaking scientists will be glad to see this translation of Cajal's great book long used as a standard textbook in the universities of Spain. The translator has followed the original text literally and with rigid accuracy. The result is somewhat unusual in nomenclature, phraseology and syntax but lacks nothing of clearness and freshness. The illustrations are Cajal's own work and are well reproduced. One chapter, on histological methods, outlines the processes used in demonstrating structural composition of tissues. An appendix gives added details of methods for preparing materials. Most of the bibliographic titles given in the book refer to the work of Cajal and his followers, so that the book, though elementary, may serve as a guide to the accomplishments of this brilliant school of Spanish histologists.

SURGICAL ANATOMY, by Grant Massie. Second Edition. 1933. Size, 6 × 9½ inches. Pages x + 458. 147 illustrations, many of which are in color. Index. Cloth. Price, $6.00. Lea & Febiger, Philadelphia.

Facts of anatomy are presented in conjunction with their clinical application. This helpful association of ideas enables the medical student to keep fresh in his mind the appearance and relations of structures studied in the dissecting room. It simplifies as far as is possible a difficult subject and presents it in a manner interesting and usable. The subject matter is divided into six general headings: the head and neck, the upper limb, the abdomen and pelvis, the thorax, and the vertebral column. The present edition has been revised to include recent developments in surgery. Additions to the work deal with injuries of the carpus, infections of the hand, surgical approaches to certain of the long bones, and the sympathetic nervous system.

ARBEITEN AUS DEM NEUROLOGISCHEN INSTITUTE AN DER WIENER UNIVERSITÄT. XXXV Band. 1933. Size, 7 × 10 inches. 181 pages. Illustrated with 57 text figures. Price, M 33. Franz Deuticke, Leipzig and Wien.

There are twelve original articles, covering different phases of neurological research at the Institute in Vienna, included in this volume.